THE DEVELOPMENT OF FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS AND HETEROPHILE HAEMAGGLUTININ ASSAYS FOR APPLICATION IN RENAL DISEASE.

by

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TO MY PARENTS

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SECTION II

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STATEMENT IN TERMS OF THE Ph.D. REGULATION 13 OF THE UNIVERSITY OF EDINBURGH

Some of the studies in this Thesis have been the subject of published articles :-

1. Preparation of Human Cells for the Assay of Serum Fibrinogen Degradation Products using Haemagglutination Inhibition.
   Hoq, M.S. and Das, P.C. (1971). Scand. J. Haemat., Suppl. 13, 101.
2. Variability of Sheep Red Cells in their Reaction to Agglutinins in Normal Human Sera.
   Hoq, M.S., Cash, J.D., Das, P.C. and Cumming R.A. (1971). Brit. J. Haemat., 21, 677.
3. Studies on a Direct Latex Agglutination Technique for the Semiquantitation of Fibrin/Fibrinogen Degradation Products.
   Hoq, M.S. and Cash, J.D. (1973). Thromb. Res., (In Press).
4. Rapid Latex - Screening Test for Urine F.D.P. Cash, J.D., Hoq, M.S., Cunningham, M. and Anderton, J.L. (1973). Lancet, 1, 153.
5. Urinary Excretion of Heterophile (sheep) Haemagglutinins and Fibrin/Fibrinogen Degradation Products following Renal Homotransplantation and in Proliferative Glomerulonephritis.
   Hoq, M.S., Anderston, J.L., Clarkson, A.R. and Cash J.D. (1973). Lancet, (In Press).

SUMMARY

The main themes of this study were 1) the development of a new T.R.C.H.I.I. (F.D.P.) in which human red cells replace sheep erythrocytes, II) the development of a new standarised assay for urinary heterophile haemagglutinins, and III) the application of the first and second technologies in glomerulonephritis and transplantation.

In the first series of studies it was discovered that glutaraldehyde was the best fixative for human erythrocytes compared to formaldehyde and pyruvicaldehyde, as these two fixatives tended to produce autoagglutination. The optimal conditions for fixation, tanning and coating were studied and defined in some detail. The fixed cells could be stored satisfactorily for at least 2 years without a reduction in the antibody titre. The sensitivity of the cells was more than a million and the test was sensitive, reliable and could detect as little as 0.5μg/ml of fibrinogen equivalent.

In the second technological study - techniques were developed which provided stable and reproducible reagents for the assay of urinary heterophile haemagglutinins. A new finding in this study was the existance of high and low reacting sheep erythrocytes and the difference in reactivity between sheep cells to haemagglutinins in normal human sera and patients' urine. The practical significane of

these results are discussed in detail. As a result of these methodological studies - a suitable glutaraldehyde fixed sheep cell preparation was obtained and the optimal conditions for the reaction ascertain for subsequent clinical application.

In the third technological study - the direct latex agglutination test (Thrombo-Wellcotest) for F.D.P. was studied. The sensitivity of this test was 4μg/ml to fibrinogen and D fragment and 0.5μg/ml to E fragment. There was a satisfactory correlation between this method and the T.R.C.H.I.I. In vitro studies, it was clearly demonstrated that the sensitivity to all digested products was good and in terms of E fragment more sensitive than T.R.C.H.I.I. using whole antifibrinogen sera. The test provides a simple rapid "one-off" bed side semiquantitation of serum F.D.P. It is new as it is sensitive to both fragment D and E, which does not apply to the existing latex technique known as the Fi-test. For urine the test was inadequate as the urine needs prior concentration and increases nonspecific agglutination reactions.

Finally, the T.R.C.H.I.I. and the heterophile haemagglutination tests were used to study urinary excretion of F.D.P. and heterophile haemagglutinins in glomerulonephritis and transplantation. These studies confirmed the concept that the origin of urinary F.D.P. in proliferative glomerulonephritis and renal transplantation is likely to be immune. Urinary sheep cells haemagglutinin were not detected in the 100 normal subjects studied nor did the F.D.P. content exceed 0.25μg/ml. There was a positive correlation obtained between the

heterophile (sheep) haemagglutinin and F.D.P. content in the urine from proliferative glomerulonephritis and following renal transplantation. Serial studies performed on individuals revealed a general tendency for periods of high heterophile haemagglutinin excretion to coincide with similar excess of F.D.P. excretion. No correlation was demonstrated between heterophile haemagglutinin titre and total protein content in the urine studied.

A correlation was recorded between the urinary heterophile (sheep) haemagglutinin, C3, IgG, IgM concentrations in the homotransplant urines, and between IgG and C3 in the proliferative and IgG in minimal lesion. In the transplant patient a rejection episode was associated with an elevation in all parameters assayed.

In a group of highly selected patients with proliferative glomerulonephritis, these parameters gave parallel information, namely the excretion of heterophile (sheep) haemagglutinin, IgG and C3. However, a patient who showed evidence of an excellent F.D.P. response to indomethacin, there was continued excretion of heterophile (sheep) haemagglutinin, IgM and C3. This patient's renal function continued to deteriorate as did the patient whose combined measurements showed no change during indomethacin administration. The patient whose renal function improved on indomethacin treatment showed a dramatic fall in the excretion of F.D.P., heterophile (sheep) haemagglutinins, IgM and C3.

In comparative studies, it was observed that rabbit erythrocytes gave an overall higher titre and detected agglutinins in a higher proportion in urines from transplant and proliferative glomerulonephritis

compared to sheep and rat cells, but in minimal lesion the sheep cells detected a greater number of positive urines than other cells. Absorption studies suggest that it is not a Paul-Bunnel type of antibody and that antibody is IgG and IgM type. The reactivity to rat cells excluded Forssman antibody.

In preliminary studies - it was observed that the techniques can be readily modified to automation using Technicon Single Channel Autoanalyser. The results are discussed in full detail.

It is proposed that the introduction of urinary heterophile haemagglutinin excretion may represent an important new tool in the future management of patients with glomerulonephritis and those following renal transplantation.

S E C T I O N 1

GENERAL INTRODUCTION

INTRODUCTION

There is now an increasing amount of experimental and clinical evidence which points to a link between immunological and coagulation mechanisms (Salmon et al, 1971; Luscher and Pfueller, 1971). Thus various diseases with an immunological pathogenesis are accompanied by the deposition of fibrin in organs, and the presence of antigen-antibody complexes may be of particular importance in the initiation of coagulation, probably through platelet and endothelial cell damage (Mustard, 1971).

The general approach to the work contained in this thesis is one in which efforts have been made to correlate immunological activity with evidence of fibrin deposition in certain forms of renal disease. The markers used have been heterophile antibodies and fibrin/fibrinogen degradation products (F.D.P.), respectively (vide infra).

CHAPTER 1.

COAGULATION

The events which lead to the formation of fibrin from fibrinogen consitutes the coagulation mechanism. It is a complex and dynamic process believed to be dependent on the activation of a series of enzyme systems, whose sequence has been tentatively documented from *in vitro* studies.

In 1771, Hewson first demonstrated that the coagulation part of the blood appeared to reside in the plasma, rather than the red cells. In 1856 Virchow postulated the existence of fibrinogen and this key

protein was isolated three years later by Denis (1859). However, it was suggested by Schmidt (1872) that at least one other factor was required which he named thrombin, and thrombin was shown by Gamgee (1879) to be a protein of the globulin variety. In what is now recognised as a series of classical experiments, Morawitz (1905) observed that tissue extracts appeared to accelerate the clotting time of plasma and that serum contained a coagulant of plasma which was not demonstrable in plasma. He concluded that blood remained fluid in blood vessels unless and until it came into contact with tissue extracts. In the presence of such extracts a precursor substance, called prothrombin, was converted to the enzyme thrombin which transformed fibrinogen to fibrin.

Although the basic principles of this theory have stood the test of time subsequent investigations and technical developments have shown it to be somewhat over-simplified. However, its basic content led Quick (1935) to develop the one-stage prothrombin time test from which he argued that the clotting time was primarily dependant upon the percursor (prothrombin) concentration. Patients were soon discovered with prolonged one-stage prothrombin times who were believed, for other reasons, not to be deficient in prothrombin. Moreover, Quick (1935) clearly demonstrated a normal prothrombin time in haemophiliac patients who had serious clinical haemostatic problems. Thus, although this original work provided the foundation for what is now known as the extrinsic coagulation pathway, these latter findings initiated studies during the period 1937 to 1954 which culminated in the realisation of an alternative pathway, now known as the intrinsic coagulation system. Since this time much effort has

been spent in attempts to define the sequence of events in which the various coagulation factors interact in both the extrinsic and intrinsic systems. Current evidence would suggest that the most important feature is the production of activated coagulation factor X which in turn plays a key role in the conversion of prothrombin to thrombin.

It has also become apparent that both systems consist essentially of a series of chain reactions in which inert stable coagulation factors are converted to active forms, which in turn act as pro-enzymes for other factors. Such a scheme, as presented in Fig. 1, was first proposed by MacFarlane in 1964 and is now referred to as the 'cascade theory' or the 'waterfall theory'. Thrombin is believed to have auto-catalytic properties at earlier sites in the cascade and this feature together with the sequential nature of the inter-reactions results in a biological amplification mechanism which results in the progressive acceleration of the reaction as it proceeds (MacFarlane, 1964). The reaction is essentially explosive in nature and is probably partly controlled by complex inhibitor systems.

CONTACT ACTIVATION

Coagulation factor XII (Hageman factor) is present in the plasma in trace amounts and has a molecular weight between a hundred to two hundred thousand. Biochemical studies would suggest that it is a glycoprotein. Although the activation of Hageman factor may be achieved by a variety of substances *in vitro*, the *in vivo* stimulus has not yet been fully elucidated, although collagen probably plays a primary role in the normal activation of factor XII in man. Factor

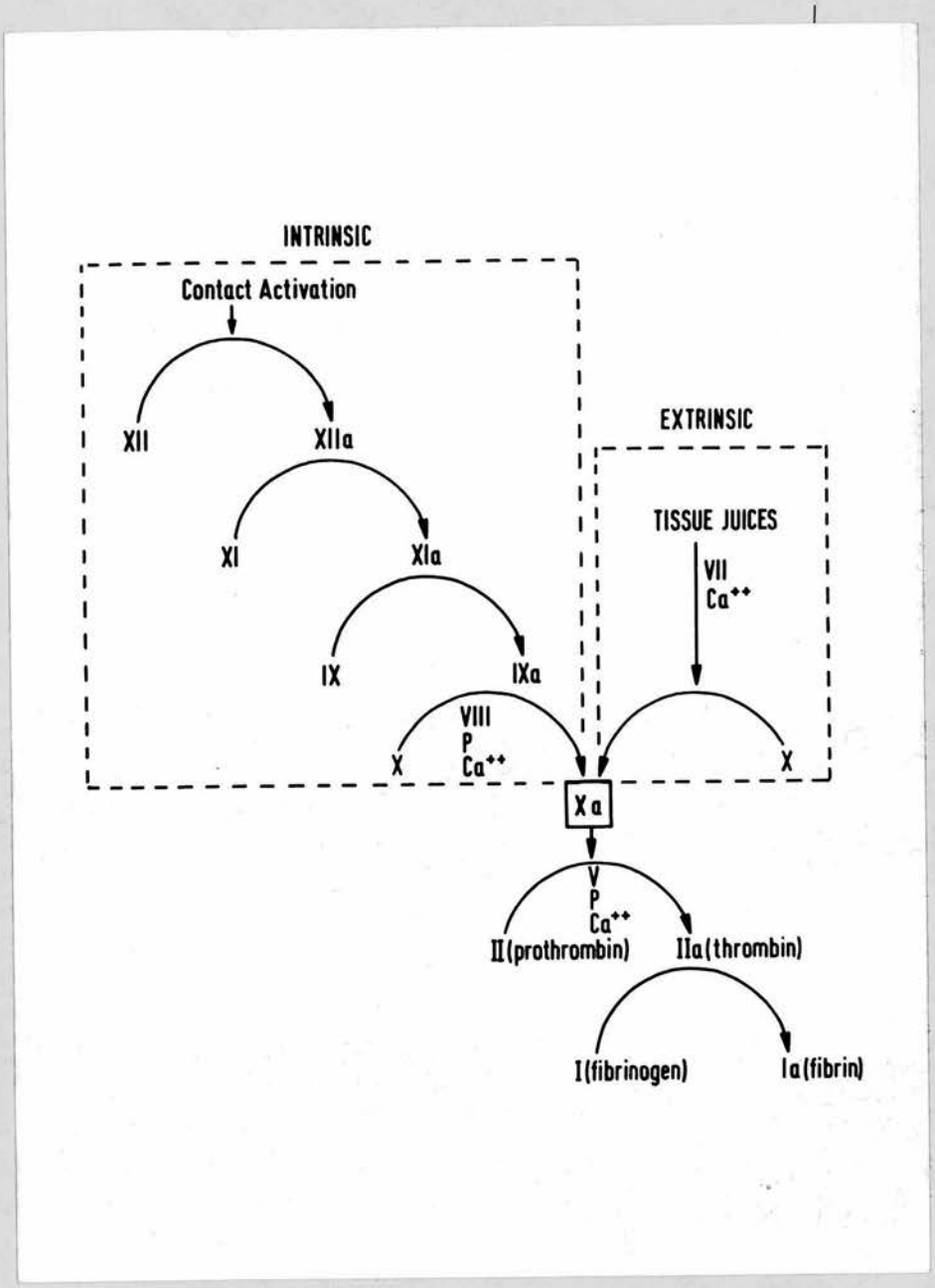

Fig. 1 Current concept of the Blood Coagulation System. The sequence of events in which various coagulation factors interact in both the extrinsic and intrinsic systems. P indicates phospholipid and Ca^{++} calcium.

XII is thought to be absorbed onto the activating surface where it complexes with factor XI which converts factor XII to its activated form (Ratnoff et al, 1961). The activation of factor XII leads to the activation of coagulation factor XI (plasma thromboplastin antecedent). Activated factor XI in turn is believed to be responsible for activating factor IX (Christmas factor) (Ratnoff and Davie, 1962).

Niewiarowski and Prou-wartelle, (1959) and Iatridis and Ferguson (1961) demonstrated increasing fibrinolytic activity of the euglobulin fraction from normal plasma when incubated with glass or in the presence of kaolin. Plasma from Hageman factor deficient patients did not show such a response in the presence of glass or kaolin. Eisen (1964) has also demonstrated that polybrene, which inhibits Hageman factor, also inhibits the fibrinolytic activity. Thus, there appears to be a link between fibrinolysis and the activation of Hageman factor. However, conflicting reports of the fibrinolytic activity of patients with factor XII deficiency would suggest that fibrinolytic activity is not triggered off solely through contact activation. Other studies have suggested that activated factor XII also plays a key role in the generation of kinins and the activation of the complement system. (Ratnoff and Miles, 1964: Pondman, 1969).

In the intrinsic coagulation system activated factor IX is complexed with factor VIII, calcium ions and phospholipid derived from platelets which converts factor X to its activated form.

TISSUE ACTIVATION

An alternative pathway for the activation of factor X is known

as the extrinsic coagulation system. It is probable that in normal physiological circumstances this system may serve as a rapid means of making available small amounts of thrombin which facilitate the activation of the slower intrinsic system. In certain pathological situations, particularly those related to intravascular coagulation in pregnancy, the extrinsic system may well be the primary mechanism underlying the conversion of fibrinogen to fibrin. The initiating event in the extrinsic coagulation mechanism is the activation of factor VII by material known as tissue thromboplastin, derived from a variety of cells. Tissue thromboplastin is a protein-phospolipid complex connected with an endoplasmic reticulum, which can split in to two components (Deutsch et al, 1964). Although the details of this system have yet to be fully worked out current evidence would suggest that tissue thromboplastin binds calcium ions and factor VII to form a complex which has enzymatic properties directed towards factor X. Factor VII is thought to be a glyco-protein which has a high affinity for binding divalent cations. It is probable that besides being of great importance in the extrinsic system factor VII, along with tissue thromboplastin, may activate factor IX in much the same way as this factor is activated in the intrinsic system. It is believed that by this route patients with Hageman factor deficiency sustain satisfactory haemostasis.

EVENTS SUBSEQUENT TO THE ACTIVATION OF FACTOR X

The conversion of prothromin to thrombin requires the presence of activated factor X, factor V, bivalent cations and phospholipid. Activated factor X lies at the heart of the coagulation cascade

mechanism and can therefore be derived from both the extrinsic and intrinsic systems. Activated factor X is an enzyme with a molecular weight of approximately 24,000 and possesses esterolytic as well as coagulant properties. Pro-thrombin is very slowly converted to thrombin by activated factor X, but, in the presence of calcium ions, factor V and phospholipid derived from platelets, the reaction is greatly accelerated. Factor V is probably a high molecular weight substance whose stability is largely dependant upon the presence of calcium ions. Neither factor V nor phospholipids either alone or together can activate prothrombin, however, it has been suggested that prothrombin is absorbed onto the phospholipid and factor V by which process its structural configuration is changed rendering it susceptible to the enzymatic action of adsorbed activated factor X, this adsorbtion process appears to be calcium dependant.

FIBRIN FORMATION

Fibrinogen is composed of three chains of peptides known as the alpha, beta and gamma chains which are linked at the end terminal by a strong disulphide knot. Thrombin acts by splitting fibrino-peptides (A and B) from the alpha and beta chains at the N-terminal ends. Thrombin has an exceedingly narrow specificity of action and hydrolyses arginyl-glycine bonds linking the fibrino-peptides to the rest of the fibrinogen molecule (Blomback and Blomback, 1969).

There is some evidence to show that the fibrino-peptides may have specific physiological roles, for they act as homeostatic inhibitors in the clotting mechanism (Silver and Murray, 1966). The fibrino-peptides may also be of some importance in the haemodynamic control of the micro-circulation as they have been shown to potentiate

the action of bradykinin and histamine (Colman et al, 1967) and affect changes in blood flow of the heart, lung and uterus when injected in exceedingly minute amounts (Bayley et al, 1967).

After the release of fibrino-peptides A and B the resultant fibrin monomer aggregates with other monomers to form a three dimensional network of fibrin polymers. This structure is unstable, soluble in urea and monochloracetic acid, but is stabilised by the action of coagulation factor XIII (fibrin stabilising factor). The end result of this interaction, in which thrombin plays a key role in the activation of factor XIII, is the production of an insoluble polymer with normal haemostatic function. These sequence of events are shown diagramatically in Figure 2.

FIBRIN MONOMER COMPLEXES

The normal polymerisation of fibrinogen may be interupted by the complexing of fibrin monomers with fibrinogen and other fibrinogen derivatives, notably fibrinogen or fibrin degradation products. The existance of such complexes was first suspected by Morrison (1946). This substance was called cryofibrinogen. Recent studies, have shown that cryofibrinogen is not simply a complex of altered fibrinogen, and Lipinski et al (1964) have demonstrated that fibrinogen degradation products, derived from the action of plasmin, complex with fibrin monomers. The most important physiological feature of these complexes is that they can interfere with normal fibrin formation and result in defective fibrin polymerisation. They may also interact with platelets and produce platelet aggregation (Wegrzynowiczet al, 1971). At the same time the aggregation of platelets releases a variety of substances,

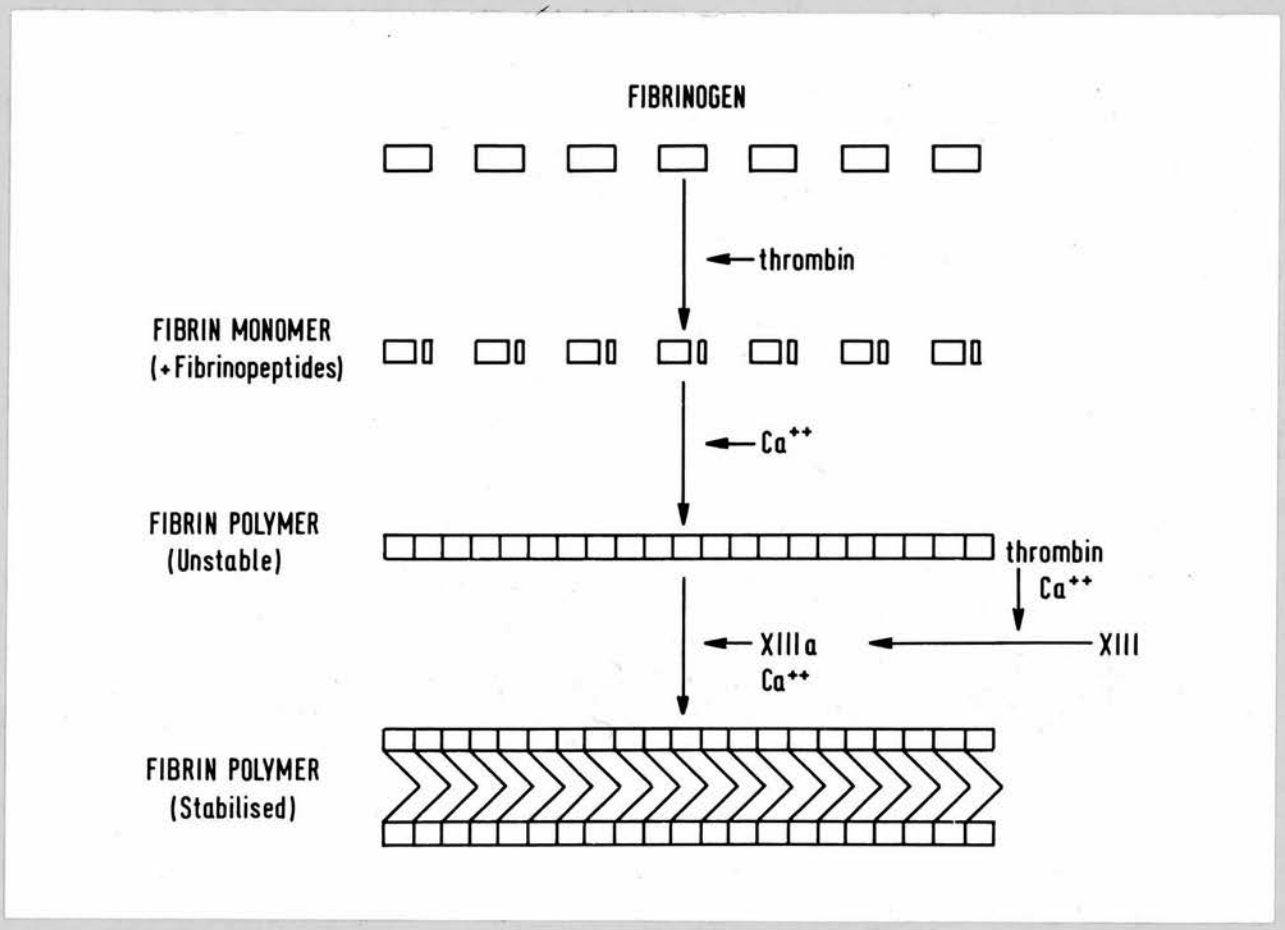

Fig. 2 A scheme of the sequence of events in the conversion of fibrinogen to fibrin.

notably platelet factor IV, which may precipitate these complexes (Niewiarowski et al, 1968).

IMMUNOLOGICAL ASPECTS OF COAGULATION

The isolated activation of the contact factors, as brought about for instance by injections of ellagic acid, does not lead to intravascular coagulation, most likely because phospholipids are the limiting factor for complete activation of the intrinsic system (Botti and Ratnoff, 1964). Essential phospholipids are provided by injured cells. The platelet is of particular interest because it is capable of making available its phospholipid under a wide variety of stimuli which leave other cells intact. However, platelets displaying PF3-activity do not induce extensive intravascular coagulation provided the contact system is not simultaneously activated.

Cell damage by immunological mechanisms giving rise to intravascular coagulation has been extensively documented (Luscher, and Pfueller, 1971). At least two mechanisms have been suggested: the interaction of cell antigens with specific antibodies and the cell damaging effects of immune-complexes. Both these reactions appear to involve complement activation (Gocke, 1965) and platelet involvement appears to be an important source of phospholipid. Damaged endothelial cells are also regarded as an essential component in some forms of these diseases. It is also possible that fibrin formation may be associated with the delayed hypersensitivity type of immunological response: one which is not thought to be concerned with antibody involvement. Such a tentative conclusion comes from the finding of high immune fibrin/fibrinogen degradation product content during renal homograft rejection (Clarkson et al, 1970).

CHAPTER 2.

THE FIBRINOLYTIC ENZYME SYSTEM

The primary role of the fibrinolytic enzyme system is believed to be concerned with the removal of unwanted fibrin in the body. Despite the original observations made by Morgagni in 1761 that blood from individuals dying suddenly, remained fluid, knowledge in this field is relatively sparse. The discovery of streptokinase, a substance derived from actively growing streptococci which facilitates the lysis of fibrin (Tillett and Garner, 1933), stimulated much research, particularly by Milstone (1941) and Christensen and MacLeod (1945). Despite this excellent start, progress has been erratic and many would consider that our understanding of the fibrinolytic enzyme system remains relatively rudimentary. This arises partly because of the paucity of satisfactory methods, but also because much of the scientific effort in this area has been devoted to the highly attractive area of thrombolytic therapy. Thus the present concepts of fibrinolysis are fragmentary and a simplified scheme of the system is shown in Figure 3.

COMPONENTS OF THE FIBRINOLYTIC ENZYME

PLASMINOGEN

This inactive precursor of plasmin is a beta-globulin contained in the euglobulin fraction of plasma. It has a strong affinity for fibrinogen, so that most purified fibrinogen preparations are contaminated with it. However, separation can be achieved by gel filtration, and purification procedures following this reveal a

protein with a molecular weight of approximately 80,000. Besides being present in the blood, it is widely distributed throughout the body tissues, excretions and secretions. The liver is probably responsible for it's production (Sherry, 1965) although immuno-flurescent studies suggest that bone marrow cells and particularly eosinophils may also contribute (Barnhart and Riddle, 1963).

The molecular weight of plasminogen and plasmin appear to be different. This is manifest by clear differences in the sedimentation and frictional coefficients and the electrophoretic mobilities. A single arginyl-valine bond is apparently split when human plasminogen is converted to plasmin by either streptokinase or urokinase. Following activation a conformational change also appears to take place and there is some evidence that suggests the splitting off of a peptide moiety during activation (Summaria et al, 1971). However, the major chemical event appears to be a single arginyl-valine bond split which results in a two chain molecule, connected by a single disulphide bond.

PLASMIN

Plasmin is relatively non-specific proteolytic enzyme which hydrolyses many proteins including coagulation factors V and VIII. It also attacks several of the components of the complement system and other plasma proteins, although it is believed that its main biological activity in normal circumstances is directed towards the digestion of fibrin. It has a molecular weight of approximately 75,000 but its precise structure has as yet not been clarified. The active site of human plasmin contains both serine and histidine residues.

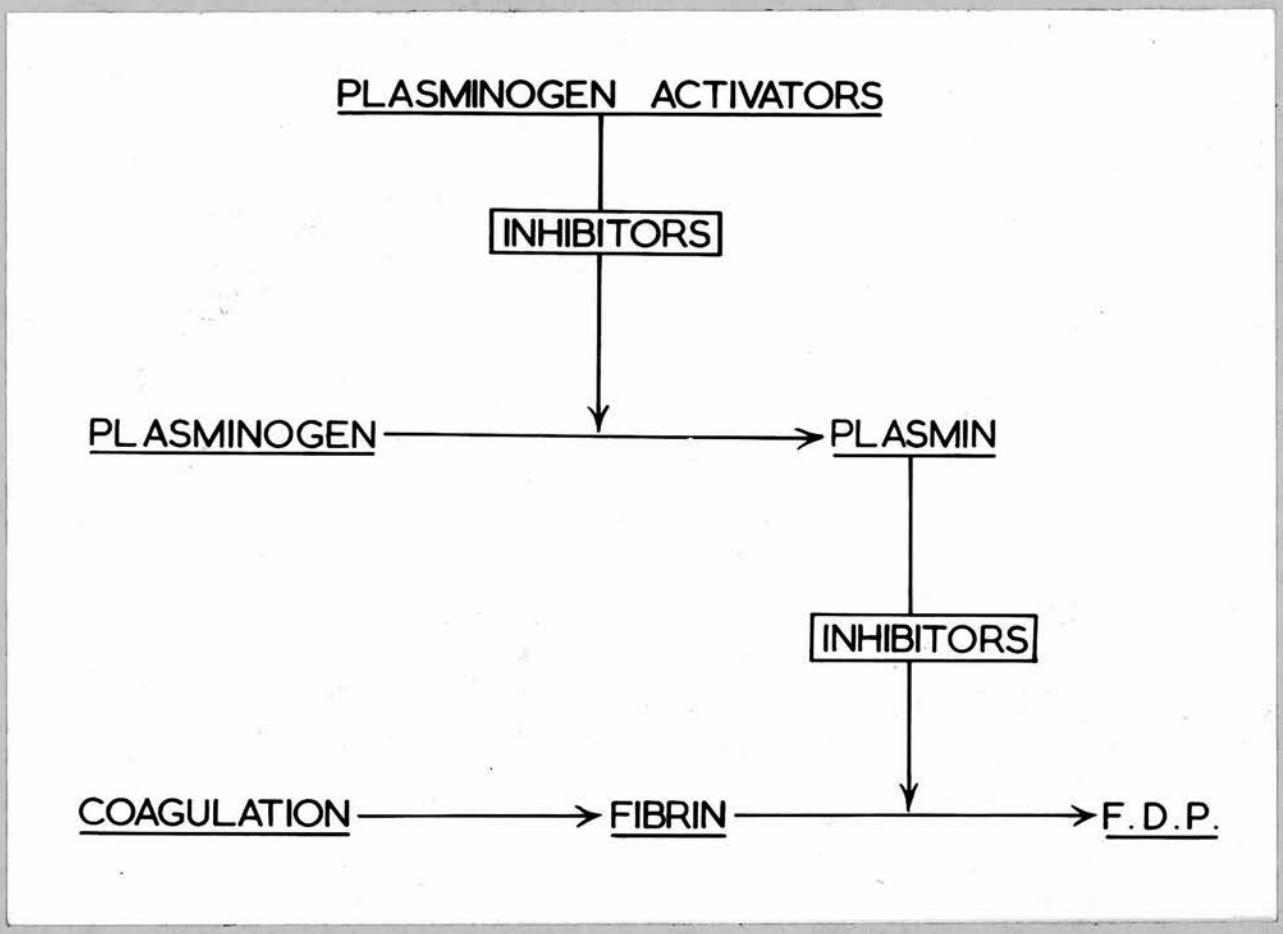

Fig. 3 A simplified scheme of fibrinolysis.

Human plasmin is similar in specificity to trypsin in that it catalyses the hydrolysis of alpha-amino substituted lysine and arginine esters. The significant actitivity, in terms of fibrinolysis appears to be that directed towards the splitting of arginine or lysine carbonyl bonds (Troll and Sherry, 1955; Beck and Jackson, 1966)

PLASMINOGEN ACTIVATORS

The plasminogen to plasmin conversion involves removal of part of the plasminogen molecule by the splitting of arginine and valine bonds by plasminogen activators. In this way "active sites" are exposed. Activators exist in two forms, some activators act directly on the plasminogen molecule and are present in blood, tissues and body fluids, secretions and excretions. Pro-activators are substances which first must be transformed to their effective form by lysokinases present in tissues, vascular endothelium and bacteria such as streptococci. Plasmin itself may also activate plasminogen by a process of autocatalysis (Alkjaersig et al, 1958)

Plasma Plasminogen Activator

When sufficiently sensitive methods are used, plasminogen activator can be detected in plasma under normal conditions. Increased levels are found under circumstances of stress, anxiety, after exertion or following adrenaline infusions. Flute (1960) was able to distinguish two types of plasma activator by electrophoresis of plasma. One was temperature stable, the other was temperature labile. The nature of these activators is unclear. The labile fraction was rapidly destroyed by heating and acid pH. Most of it was precipitated in the euglobulin fraction of plasma

and advantage is taken of this in the commonly performed assay for plasma activator, the euglobulin lysis time. Negligible amounts are found in serum euglobulin (McFarlane and Pilling, 1946) as it is adsorbed on to fibrin during clotting (Lassen, 1958). From a practical point of view the lability of plasma activators cannot be overemphasised.

There now seems little doubt that the blood vessel walls are the major source of plasma activator as originally suggested by Nolf (1904). Vascular connective tissue was noticed to have high activator content by Astrup (1961) and Kwaan and Astrup (1964), but the use of the technique of fibrinolysis autography developed by Todd (1958) has demonstrated that the intimal surfaces of veins, venules and capillaries are largely responsible for its production (Todd, 1959, 1964; Warren, 1964). It is possible that an especially large contribution of activator in the circulation is contributed by specific organs such as the kidney (Buluk and Furman, 1962; Niewiarowski et al, 1964) and uterus (Maki et al, 1965).

The physiological control of plasma activator release has recently attracted much attention. However, as yet, little is known about the factors controlling the release and maintenance of circulating levels. There is diurnal variation in plasma activator concentration with the peak during the day and a trough at night (Fearnley et al, 1957). It is probably degraded in the liver and lungs and may be excreted in the urine.

Many physiological and pharmacological stimuli increase the circulating activator content. Kwaan and McFadzean (1956, 1957) suggested that its release may be under neurological control.

Following this postulate numerous attempts were made to study this aspect of its control but the results were controversial and contradictory as it was found that there was marked variability from individual to individual both of resting levels and response to stimulation. Moreover, reproducible stimuli were rarely used. A new approach to this subject was introduced by Cash (1966) and Cash and Woodfield (1968) who, by using standardised exercise procedures, demonstrated that the fibrinolytic reactivity in any one individual when expressed as a percentage increase of the resting level was reproducible, but was variable between individuals. In particular, a group of 'poor responders' was isolated. These authors concluded that if rapid dynamic changes of fibrinolysis are required the ability of the individual to augment the concentration of plasminogen activator could be an important factor in maintaining homeostasis (Cash and Woodfield, 1967). Further studies of the systemic plasminogen activator response to catecholamines revealed that it is probably derived from two separate components (Cash, Woodfield and Allan, 1970) which may share the same adrenergic receptor site. A relatively minor one may be secondary to vasoactive changes, whereas the second major component is quite independant of this phenomenon.

Tissue Activator

Most tissues of the body contain a plasminogen activator which is localized in the microsomes of cell cytoplasm. Unlike plasma activator it possesses marked stability to heat, drying, acidification and chloroform treatment and is bound firmly to structural proteins.

Extraction and partial purification have been achieved due to its solubility in potassium thiocyanate (Astrup and Stage, 1952) thus allowing quantitative determination of its concentration in various tissues. It may be released from cells after tissue injury. Little is known of the chemical properties of tissue activator, but it behaves in a different way to streptokinase.

Its physiological role is probably concerned with the maintenance of tissue repair. Fibrin participates in tissue repair by serving as a matrix for the formation of reparative connective tissue. Tissue activator may be of biological significance in the prevention of excess reparative tissues, as resolution and removal of the fibrin appears to be required to re-establish normal structure.

Urokinase

Fibrinolytic activity of the urine was recognised by MacFarlane and Pilling (1947) and an activator of plasminogen - urokinase - was demonstrated by Williams (1951). Urokinase has now been purified and seems to activate plasminogen to plasmin by first order kinetics. Its molecular weight is approximately 50,000. Its role in the regulation of endogenous fibrinolysis has been difficult to evaluate due to the difference of opinion as to its origin. As the content in renal pelvic urine is the same as excreted urine (Bjerrehuus, 1952), it seems unlikely that the lower urinary tract contributes significantly to its production. Urokinase excretion is increased after physical exercise and cardiac surgery, and is low in renal insufficiency, cardiac failure, and carcinomatosis. The work of Kucinski et al, (1968) showing an immunological difference

between plasma activator and urokinase would suggest that its origin is exclusively renal. However, the intrarenal site of urokinase is not clear. Using fibrinolysis autography, activator activity can be detected in glomerular and peritubular capillaries of the renal cortex, but its greatest concentration is in the medulla where presumably it is concentrated. Recent work with tissue cultures has suggested that a soluble activator probably originates in the juxtamedullary apparatus (Painter and Charles, 1962; Prokopowicz et al, 1964).

The physiological function of urokinase is also uncertain. Its primary role may be concerned with the maintenance of the patency of renal tubules and the lower urinary tract.

Activator in other body fluids

Activators of plasminogen are present in tears, saliva, milk, seminal fluid and cerebrospinal fluid. Their physiological importance is unknown, but may be much the same in principle as urokinase.

Non-physiological activators

Streptokinase identified in 1933 has been purified for use in thrombolytic therapy. Plasminogen is not activated by streptokinase unless proteins from the globulin series are also present (Mullertz and Lassen, 1953). From this was inferred the presence of a pro-activator in human blood.

Other non-specific substances which may cause activation *in vitro* include peptones, urea, heparin and protamine while liquid cultures of Aspergillus oryzae and cell free filtrates of staphylococci contain plasminogen activators.

INHIBITORS OF FIBRINOLYSIS

One of the control mechanisms of fibrinolysis is believed to be concerned with inhibitors against activators and plasmin. In addition a great number of inhibitors of exogenous origin are known.

Physiological antiactivators

Inhibitors of activator may be essential for fibrinolysis regulation, but their separate identity from anti-plasmin is still questioned, as although some evidence exists for their presence it is not yet absolutely conclusive. The formation of an anti-activator during incubation of blood in glass was described by Flute (1960) and found to be enhanced by calcium ions. The existence of antiactivator has recently been reinforced by the work of Bennet (1970). Thus it seems probable that the anti-activator in plasma plays an important role in reversibly complexing with circulating activator, thus maintaining a low level of free plasminogen activator in solution.

Physiological antiplasmins

There are at least four antiplasmins in blood. The alpha - 1 and alpha - 2 antiplasmins in the globulin fraction are the best characterised. Alpha - 2 antiplasmins, with molecular weight of 845,000 is an immediately effective competitive inhibitor of plasmin, while the alpha - 1 antiplasmin has a molecular weight of 47,000 reacts slowly by forming a stable complex with plasmin (Summaria, et al, 1971). The trypsin inhibiting capacity of plasma, has only negligible activity against plasmin. Another anti-plasmin may exist in the gamma globulin fraction of plasma proteins (Morian et al,

1964). Platelets also possess antiplasmin activity and this may be of importance in the resistance to clot lysis, in vivo.

Miscellaneous exogenous antifibrinolysins

Several unrelated substances are known to inhibit plasmin action directly or indirectly by virtue of antiactivator activity. These include soya-bean trypsin inhibitor, basic amino acids, heparin, heavy metals, trasylol, epsilon amino caproic acid (E.A.C.A.), amino-methyl cyclohexane carboxylic acid (A.M.C.H.A.) and para-amino methyl benzoic acid (P.A.M.B.A.).

THE PLASMIN DEGRADATION OF FIBRINOGEN AND FIBRIN

During the degradation of fibrinogen and fibrin by the fibrinolytic enzyme plasmin, the substrate molecule is split into several soluble polypeptide fragments known as fibrin/fibrinogen degradation products (F.D.P.). The nature of the products formed in vitro, by this reaction depends on the time it is allowed to proceed. Early lysis products are of high molecular weight, remain clottable with thrombin, possess powerful anticoagulant properties and are still susceptible to further lysis by plasmin. Continued lysis results in smaller molecular weight non-clottable fragments, with negligible anticoagulant action and resistance to further lysis.

Characterisation of F.D.P.

Five distinct fractions, name A,B,C,D and E were originally obtained by Nussenzweig et al (1961) after subjecting fibrinogen digests to D.E.A.E. cellulose chromatography. Two of these D and E precipitated with anti-fibrinogen serum but the remainder appeared

to have lost antigenic similarity during digestion. Fletcher et al (1962, 1966) found that only D and E fragments could be reliably produced with prolonged digestion and these workers were able to estimate the molecular weight of these plasmin resistant fragments at 88,000 and 33,000 respectively. On the other hand Jamieson and Pert (1963) using starch gel electrophoresis found 10 different bands.

From observations based on the biological and physical properties of F.D.P., Marder, Shulman and Carroll (1969) have suggested that the lysis of fibrinogen and fibrin is a sequential process in which three distinct phases are apparent. By using immuno-electrophoresis, ultracentrifugation and gel filtration techniques they were able to characterise the nature of the products formed at each stage of digestion. Moreover, by introducing pevikon-block electrophoresis they separated sufficient quantities for purification, concentration and subsequent immunological studies (Marder, James and Sherry, 1969). In the first stage, a number of small molecular weight peptides are rapidly split from the fibrinogen molecule which has a molecular weight of 300,000, thus leaving a single, large clottable fragment of M.W. 240,000 - 270,000, which was termed fragment X. The second stage appeared slower and the sequence of events less clear than in the first. The clottability of the products is lost and the anti-coagulant properties increased as measured by prolongation of the thrombin time. It is Marder's view that during the second stage, fragment X is cleaved into two giving rise to fragments with molecular weights of 155,000 and 90,000 called fragments Y and D

respectively. The third stage is the slowest and results in the formation of plasmin resistant fragments named D and E (M.W. 30,000 - 50,000).

There still remains some disagreement concerning the proteolytic reaction, especially the second and third stages. For example Fletcher et al (1966) proposed that further lysis of fragment X resulted in a large number of products of progressibley smaller molecular weights. However, subsequent work with G-200 Sephadex column chromatography provided substanial evidence for the existence of two distinct intermediate degradation products (Marder, Shulman and Carroll, 1967, 1969) and does not lend support to the view that numerous intermediate products other than fragment X appear during plasmin digestion. It thus seems that plasmin causes an asymmetrical fragmentation of the fibrinogen molecule, as represented in Fig. 4.

One of the interesting features of this work is that little is known about the details of fibrin degradation. It has been assumed that this is likely to be identical to fibrinogen. This assumption may not be entirely correct.

Biological Properties of F.D.P.

Products formed in the early stages of fibrinogen degradation are strongly anticoagulant, a property originally attributed to inhibition of the action of thrombin (Triantaphyllopoulos, 1958; Niewiarowski and Kowalski, 1958). However, with increased understanding of the fibrinogen to fibrin conversion, it is now possible to attribute other anticoagulant properties to first and second stage

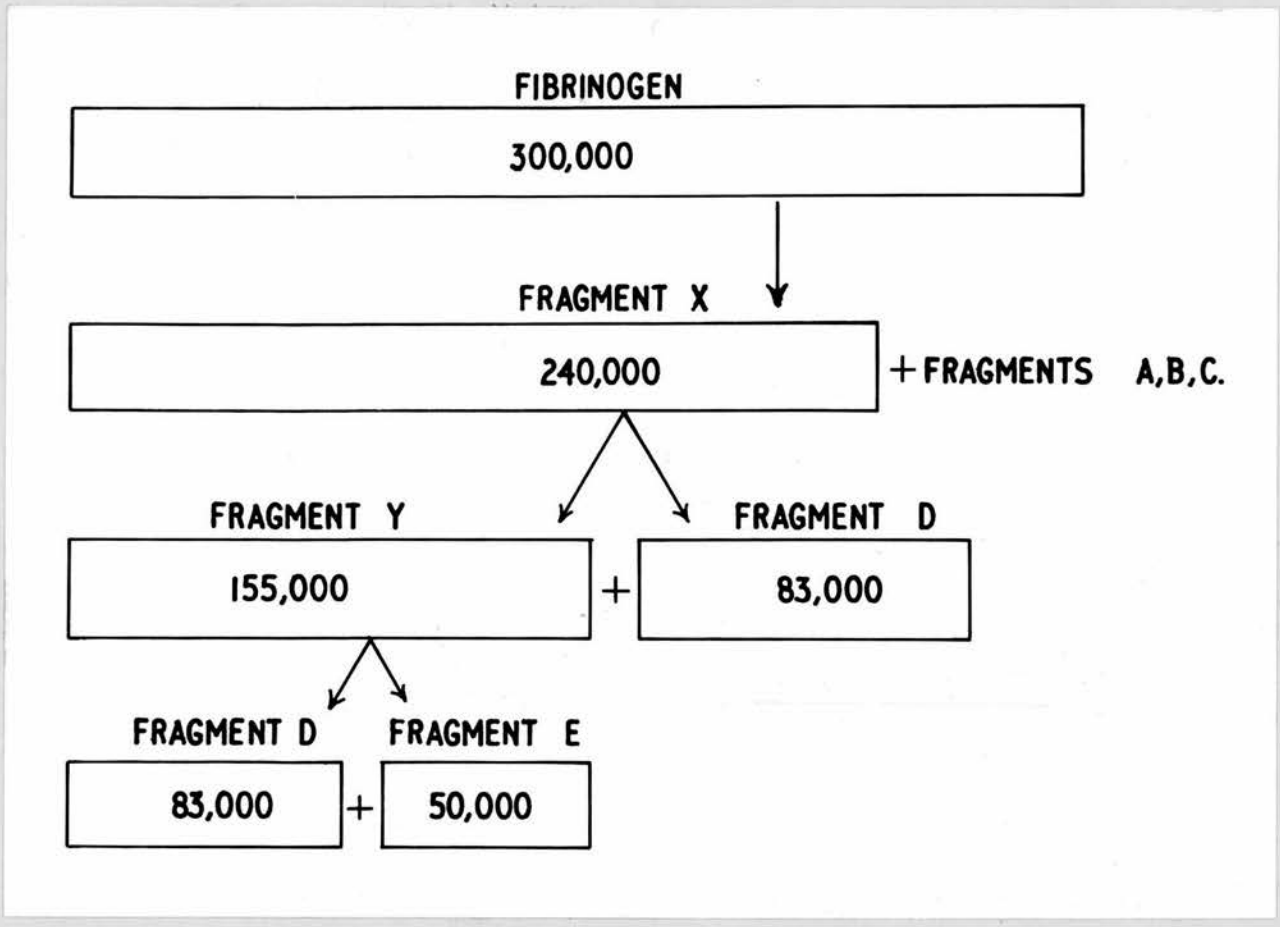

Fig. 4 Schematic representation of the asymmetrical fragmentation of the fibrinogen molecule by plasmin (after Marder, V.J., 1971).

F.D.P. Polymerisation of fibrin is inhibited (Alkjaersig et al, 1962; Latallo et al, 1962) as F.D.P. complexes with normally formed fibrin polymers to form abnormal polymers of diminished tensile strength (Bang et al, 1962; Hirsh et al, 1965) which may themselves be incoagulable (Lipinski et al, 1967). They also complex with fibrinogen, fibrin monomer and activated coagulation factors (Kowalski, 1960; Miller and Sanchez-Avalos, 1968), and may inhibit platelet aggregation (Kowalski et al, 1964; Larrieu et al, 1966) as previously mentioned. This anticoagulant action is of obvious importance in clinical situations of excessive fibrinolysis secondary to intra-vascular coagulation, where a life-threatening bleeding tendency sometimes develops.

F.D.P. may also possess actions of pharmacological importance in that they potentiate the hypotensive effect of bradykinin, and hypertensive effect of angiotensin in the rat, and the action of adrenaline on the rat myometrium (Malofiejew, 1971).

Quantitation of F.D.P.

F.D.P. were originally detected by measurement of their anticoagulant effect (Worowski et al, 1964) but this method is non-specific and has proved to be insensitive. The introduction of immunological methods for the quantitation of F.D.P. has created great interest, as it may provide an approach to assess actual *in vivo* fibrinolysis. Anti-fibrinogen serum has been employed in all immuno-assays for F.D.P. as they retain certain antigens common to native fibrinogen. Such methods as precipitation by flocculation (Ferreira and Murat, 1963) immuno-electrophoresis (Nilehn and

Nilsson, 1964) and radio-immunoassay (Catt et al, 1968) have been used, but the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) has gained widest popularity (Merskey et al, 1966). With the T.R.C.H.I.I. accurate semiquantitation of F.D.P. content has become a reality. More recently a non-immunological method has been introduced, based on the ability of fibrinogen and certain F.D.P. to clump specific strains of staphylococci (Allington, 1967; Hawiger et al, 1970). This method also provides a sensitive quantitative assay for F.D.P. and it has been compared with the T.R.C.H.I.I. by several groups (Thomas et al, 1970; Thuot and Larrieu, 1971). Both tests are equally sensitive to fibrinogen and higher molecular weight early derivatives. However, the T.R.C.H.I.I. is far more sensitive to the lower molecular weight products D and E. Certain technical difficulties still remain with the T.R.C.H.I.I. and above all, the method is somewhat laborious. These aspects will be dealt with in more detail in the technical developments section of this thesis.

Clearance of products of fibrinolysis

The mechanisms available for clearing the products of fibrinolysis include the reticulo-endothelial system, neutrophil leukocytes and the kidney. On the basis of immunoflurorescent studies in rabbits after infusion of endotoxin or thrombin, the reticulo-endothelial cells of the liver and spleen were implicated in the removal of fibrin (Lee and McCluskey, 1962). In states of low grade intravascular coagulation it was suggested that the R-E system removed the bulk of fibrin. Under certain circumstances however,

neutrophils contribute to this removal (Barnhart and Cress, 1967). It also seems that when renal damage is present, urinary excretion also takes place (Clarkson et al, 1971).

IN VIVO FIBRINOLYSIS

Several theories have been advanced to explain the mechanism of thrombolysis by plasma activator *in vivo*. Each has taken into account the fundamental observations that the dissolution of fibrin can occur without the appearance of free plasmin in the circulating blood, i.e. fibrin is attacked but fibrinogen is spared. Deposition of fibrin provides a focus for fibrinolysis and Fearnley (1953) and Sherry et al (1959) have proposed that activator diffuses into the interstices of the clot, is actively adsorbed on to fibrin, and converts plasminogen, entrapped with its closely associated fibrinogen, into plasmin. Anti-plasmins diffuse into the thrombus less readily. Local lysis thus ensues and any free plasmin managing to escape is neutralized by circulating anti-plasmins. A different view was put forward by Ambrus and Markus (1960) who suggested that fibrin is able to dissociate plasmin from a plasmin-antiplasmin complex. Wolf (1968) postulated that plasminogen activator and anti-activator exist as a loosely formed complex which dissociates during the diffusion of plasma through a thrombus. As anti-plasmins and anti-activators diffuse less rapidly due to their large size asymmetrical shape, activator is freed and plasminogen activation occurs unimpeded. While each theory is attractive they are not necessarily mutually exclusive. However, further work is necessary before firm conclusions can be drawn.

CHAPTER 3.

HETEROPHILE HAEMAGGLUTININS

Serum factors agglutinating red blood cells of another species are called heteroagglutinins. They were first discovered by Landois (1875) but were investigated in more detail by Landsteiner (1900). They are best defined as antibodies combining with a quite different antigen than used for immunisation. Heteroagglutinin(s) in normal human sera against sheep erythrocytes have since been extensively studied. Stuart et al (1935) found agglutinins against sheep red cells in 63.8 per cent of 760 samples studied; Bunnell (1933) 56.3% out of 1600 sera. The titres varied from 16 to 400. Paul and Bunnell (1932) and Davidsohn (1933) noted a correlation of the titre and the age of the subject: finding low titres among infants and children, with a gradual increase to the age of 10 years. Thereafter the titre fell with increasing age. The titres were significantly higher in females than in males.

The agglutination of other animal erythrocytes in normal sera has not been studied quite as extensively. However, the studies of Salo (1966) demonstrated that the titres are highest in rabbit and descend in the following order: guinea pig, horse and sheep. As a rule chicken erythrocytes are not agglutinated by normal human sera.

In 1932 Paul and Bunnell reported a study of 275 sera from patients with various diseases. High agglutination titres against sheep erythrocytes were found in four cases of infectious mononucleosis (I.M.). This observation has since been confirmed by many investigators. The anti-sheep agglutinin in sera from patients with I.M. differs significantly from that found in normal sera. Papain

treatment of sheep cells increases the titre of anti-sheep agglutinins in normal sera, whereas it has little or no effect on the titre of I.M. sera.

DEAE-cellulose chromatography, gel filtration on sephadex G 200 and 2-mercaptoethanol treatment have suggested that the bulk of the heterophile agglutinin in normal sera is IgM. However, there appears to be a small IgG component (Salo, 1966).

The heteroagglutinins in normal sera are regarded as a group of cross-reacting antibodies. Thus the agglutination titres against rabbit red cells have been reported to be low in sera with low agglutination titres to sheep erythrocytes. The same correlation is shown for high titre samples (Stuart et al, 1935). Early studies suggested that the same antibody reacted with different animal red cells because of the presence of a common partial receptor on the cell surface. Subsequent absorption studies would support the principle of a partial common receptor.

In 1911 Forssman produced high titres of sheep haemolysins and agglutinins by immunisation of rabbits with aqueous suspensions of guinea pig organs. This observation initiated an intensive period of investigation of the occurrence, distribution and nature of these types of heterophile antigens, i.e. antigens shared by unrelated species. Forssman antigens have been found in the tissues of many mammals, birds and fish as well as a wide spectrum of micro-organisms. Of specific interest to the work reported in the present study is that although the sheep is Forssman antigen positive, the rat is negative as is man.

A multiplicity of other heterophile antigens have been discovered

since 1911 and the existance of similar antigens in micro-organisms and mammalian tissue has been proposed in some infectious diseases, notably serum sickness and infectious mononucleosis. Recent studies have demonstrated cross-reactions between group A streptococcal antigens and mammalian tissue components, and between pneumococcal polysaccharides and the transplant antigens of certain strains of mice. Moreover, heat-killed group A streptococci induce in mice, rats, guinea-pigs and rabbits a state of hypersensitivity to skin allografts which is indistinguishable from that resulting in pre-treatment of the recipients with allogeneic tissue. Thus it has been suggested that the stimulation of the heterologus antigens may operate in conditioning the hosts response to tissue transplants.

An alternative explanation for the production of heterophile antibodies has been the suggestion that some morbid processes may result in alterations of autologous antigens to the point where they resemble foreign antigens. These altered antigens then stimulate the formation of antibodies which combine with cross-reacting foreign antigens. It has been shown that rabbits injected with autologous but denatured gamma globulin, formed antibodies which reacted with foreign gamma globulin (McCluskey et al, 1962). A similar mechanism has been postulated for the marked increase in heterophile antibody titre following thermal injury in animals and patients (Kano et al, 1967).

Both mechanisms have been invoked by Iwasaki et al, (1967) Rapaport et al (1968) and Kano and Milgrom (1970) to explain their findings of a significant increase in serum heterophile antibody titre to sheep, guinea-pig and rat erythrocytes coincident with an

episode of renal allograft rejection. Although these observations have not been confirmed by O'Kane et al (1969), marked increases in heterophile antibody to rat erythrocytes have been recorded following skin grafts (Etheredge and Najarian, 1970) and to sheep erythrocytes during serum sickness (Davidsohn, 1930).

The importance of these various observations in clinical situations are considerable. In many of the conditions in which tissue damage related to immunological injury occurs F.D.P. are currently being investigated; also the use of the T.R.C.H.I.I. in which sheep cells are used could lead to a serious misinterpretation of the data in those samples with high titre heterophile antibodies. The possibility also exists of examining whether a correlation can be found between urine F.D.P. content and the heterophile antibody content as potential markers of coagulation and immunological activity respectively.

CHAPTER 4.

COAGULATION AND RENAL DISEASE

During the last 15 years increasing evidence has accumulated which suggests that intravascular coagulation may be associated in the natural history of certain renal disorder. The evidence is based mainly on immuno-fluorescent and electron microscopic studies and on changes in circulating coagulation factors accompanying some specific diseases in man, but particularly in experimental animals. It was widely recognised before the introduction of the electron microscope and immunological staining procedures that a substance commonly referred to as fibrinoid was present within glomeruli in several types of acute glomerular diseases, such as acute glomerulonephritis, toxaemia of pregnancy, glomerulonephritis associated with systemic lupus erythematosus, polyarteritis nodosa, thrombotic thrombocytopenic purpura and experimentally induced hypersensitivity states in animals. Fibrinoid was also noted in a large variety of pyogenic and other inflammatory and degenerative lesions, but much controversy existed as to its nature and origin. Part of the controversy arose from differing staining properties of this material when subjected to specialised staining procedures which were unable to discriminate between degenerating collagen, altered ground substance, and deposited fibrin. However, the development, by Coons and Kaplan (1950), of a method for the detection and localisation of antigen by means of fluorescent antibodies provided the basis for a more accurate assessment of the nature of fibrinoid.

Fluorescein labelled human anti-fibrinogen serum prepared in rabbits was used by Gitlin, Craig and Janeway (1957) to study fibrinoid in biopsy and autopsy specimens from patients with rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, glomerulonephritis, polyarteritis nodosa and thrombotic thrombocytopaenic purpura (Craig and Gitlin, 1957). Their findings indicated that in these lesions the fibrinoid and hyaline material was composed, at least in part, of fibrin. Moreover, the extent of fibrin deposition was found to be much greater than revealed by conventional staining methods, (Gitlin and Craig, 1957). Similar views were expressed by Lendrum et al (1962) after a meticulous study of vascular lesions by standard and personally developed staining procedures. The reasons and mechanisms for the fibrin deposition was not, however, pursued.

Vassalli, Simon and Rouiller (1963) performed a logical series of experiments by inducing intravascular coagulation in animals and studied the electron microscopic lesions within the glomeruli. Three substances thought to activate the coagulation process by different means were infused intra-aortically into separate groups of rabbits: liquoid (sodium polyanetholsulphonate), whose action was presumed to be mediated via platelets, thrombin which promotes the conversion of fibrinogen to fibrin, and thromboplastin an activator of prothrombin via the extrinsic coagulation system. The ultrastructural appearances were similar in each group; not only did glomerular thrombosis occur, but a variety of abnormalities commonly present in human renal diseases were observed. Moreover, in a significant number of animals sacrificed later, progression to glomerular sclerosis had occurred. A most striking finding was the

variation in extent and severity of the lesions produced in different animals by similar stimuli. More severe abnormalities were consistently seen in animals simultaneously infused with a fibrinolytic inhibitor, such as epsilon amino caproic acid (E.A.C.A.).

These observations were extended to other experimental models which more closely approximated human immunological renal diseases. Using a form of Masugi glomerulonephritis (Masugi, 1934) induced by the injection of rabbit anti-kidney serum produced in sheep, Vassalli and McCluskey (1964) demonstrated glomerular lesions similar to those seen after intravascular coagulation. During the latent phase of five days only minor glomerular endothelial cell swelling was observed associated with the deposition of sheep γ-globulin along the basement membrane. For the next 10-15 days glomerular inflammatory reaction of increasing severity was seen. Severe proliferative changes uniformly distributed through all glomeruli often with crescent formation and polymorph infiltration gave way after 15 days to progressive glomerular sclerosis. Rabbit γ-globulin and fibrin were present along the basement membrane in the acute phase. Fibrin was also seen in the capillary lumina and Bowman's space. In animals treated with the oral anticoagulants many of these histological and immuno-fluorescent abnormalities did not develop.

It was concluded that :

1. The immune reaction within the glomerulus initiated the coagulation process and resulted in the formation of fibrin and fibrinoid,
2. The fibrinoid consisted of γ-globulin (and presumably complement) as well as fibrin,

3. The phagocytosis of this material by endothelial, mesangial and epithelial cells was responsible for their swelling and proliferation,
4. The coagulation process was an essential factor in the development of progressive sclerosis.

Much of this animal work has subsequently been confirmed by other workers. Humair, Potter and Kwaan (1969a, 1969b) produced similar abnormalities in rats by intravascular coagulation and with a form of Masugi nephritis. However, urokinase, a plasminogen activator occuring naturally in the urine, was found to be superior to anti-coagulation in preventing the inflammatory glomerular lesions.

Thus, in these experimental models, lesions were produced in the kidneys which closely resembled glomerulonephritis, and renal graft rejection as seen in humans. Their relevance to man, however, has yet to be determined.

Since the introduction of more routine immunofluorescence techniques to the study of human renal biopsy material, the presence of fibrin in diseased glomeruli has become increasingly recognized. Following the initial adaptation of the method of Coons and Kaplan (1950) to renal tissue (Mellors and Ortega, 1956) other observations have concerned the role of immunoglobulins and complement components in the pathogenesis of glomerulonephritis. Two generally accepted theories as to its causation have been proposed by Dixon (1968), once again on the basis of animal experimentation. The fluorescence pattern of γ-globulin and complement takes one of two forms. In the first there is a uniform linear distribution along the glomerular capillary basement membrane which is attributed to the binding, at

this site of antibodies against glomerular basement membrane (anti-G.B.M. antibodies) with the specific antigen. In the second type freely circulating antigen-antibody complexes lodges within the basement membrane and gives rise to a characteristic granular or lumpy appearance. While discrete examples of each type of glomerulonephritis have been described in humans, in the large majority of cases no such distinct pathogenetic mechanism is found.

Fibrin has been found frequently using immuno-fluorescene in human glomerulonephritis (Sturgill and Westervelt, 1965; Michael et al, 1966) in rejecting human renal homotransplants (Busch et al, 1967; McKenzie and Whittingham 1968), but it has also been found in many other, presumed non-immunological renal lesions and thus it must be regarded as a non-specific feature of glomerular damage. Although a role of fibrin deposition in the natural history of glomerulonephritis and renal homograft rejection remains a matter of debate, some nephrologists have been impressed with the evidence and have used anticoagulation to combat its local formation. This form of therapy has abundant precedent in laboratory animals (Silverskiold, 1940; Kleinerman, 1954; Vassalli and McCluskey, 1964) has usually taken the form of heparin infusion, oral dicoumarol or warfarin administration. Encouraging results have been obtained, and in man, Kincaid-Smith, Saker and Fairley (1968) have used anticoagulation in glomerular diseases which often gives rise to rapid and permanent loss of renal function. The same group (Kincaid-Smith, Laver and Fairley, 1970) are also enthusiastic about the benefit derived from such therapy in other forms of proliferative

glomerulonephritis, and in human renal homotransplant rejection (Kincaid-Smith, 1970). Much of this work has been done in an uncontrolled fashion and where more rigidly controlled studies have been reported, the results are not as impressive although in occasional cases dramatic response seems to occur (Herdman et al, 1970; Arieff and Pinggera, 1972).

Following the successful development of a T.R.C.H.I.I. for F.D.P. determinations using human erythrocytes, which forms part of the work contained in this Thesis (vide infra), Clarkson et al (1970, 1971) were able to show that intraglomerular fibrin deposition was a constant feature in certain forms of glomerulonephritis and during renal homotransplant rejection. No information, however, was obtained during these studies to ascertain the mechanisms involved in this process, and specifically whether evidence of immunological tissue damage was the primary cause of the fibrin deposition.

CHAPTER 5.

AIMS OF PROJECT

Despite the success of the early studies on the urinary F.D.P. content of urine following renal homotransplantation (Clarkson et al, 1970), in his laboratory, it was clear that certain technical difficulties existed : in particular the presence of urinary agglutinins to the sheep red cells used for the T.R.C.H.I.I. Occasionally they proved difficult to absorp and at all times it was necessary to run an absorption procedure before the F.D.P. assay could be undertaken. This meant that the urinary F.D.P. assay was laborious and time-consuming, and not ideally suited for the planned extensive serial studies on a large group of patients with glomerulonephritis. The question also arose as to origins of the urinary heterophile (sheep) haemagglutinin. Was it related to the immune process or simply reflected the degree and type of proteinuria? Was it a more specific index of immune glomerular damage than F.D.P.?

The essential aims of the work contained in this Thesis were directed towards these problems. In the first place studies were undertaken to develop a T.R.C.H.I.I. for F.D.P. using human red cells which could be stored, as the sheep cells, for prolonged periods of time. By using homologous cells the problem of the heterophile haemagglutinin would be evaded. Other approaches to the assay of urinary F.D.P. were also examined. In the second place studies were designed to establish an optimum assay for the quantitation of heterophile haemagglutinins in urine. Finally, if successful,

these assays were to be run in parallel in a preliminary investigation of the urinary F.D.P. and heterophile haemagglutinin content of urine in healthy controls and patients with glomerulonephritis and those following renal homotransplantation.

SECTION II

TECHNICAL STUDIES

CHAPTER 6.

STUDIES ON THE PREPARATION OF PRESERVED AND SENSITISED HUMAN RED CELLS FOR THE ASSAY OF SERUM FIBRINOGEN/FIBRIN DEGRADATION PRODUCTS.

INTRODUCTION

The original tanned red cell haemagglutination technique, devised by Merskey et al (1966), made use of sheep red cells on to which human fibrinogen had been attached. This led to the problem of absorption of sheep cell agglutinins before each assay. One approach to circumnavigating this problem has been to investigate the possibility of finding low reacting sheep red cells (vide supra), the more direct approach, when analysing human sera for F.D.P. would be to replace sheep by human red cells. Merskey et al (1969) introduced human red cells for the T.R.C.H.I.I. but it has been shown to be a particularly difficult adaptation, due to the high incidence of auto-agglutination when preserved in the usual way with formaldehyde. This problem has proved to be so great that workers using human red cells have been forced to avoid the preservation stage. Thus it has been necessary to prepare, at weekly intervals, new batches of tanned and sensitised cells. Such an approach is exceedingly time-wasting and makes standardisation tedious and difficult. The following section deals with attempts to make available human red cells for the T.R.C.H.I.I. with long term storage characteristics similar to the sheep cell F.D.P. assay.

The preparation of sensitised human red cells can be conveniently divided into three separate procedures : (a) red cell fixation, (b) treatment with tannic acid and (c) sensitisation. These three features have been the subject of separate detailed investigations and will be discussed as such.

RED CELL FIXATION STUDIES

The attachment of proteins to intact erythrocytes can be achieved by means of specific coupling agents such as bisdiazobenzidine (Pressman et al, 1942; Hamashige and Arquilla, 1963), or tannic acid (Boyden, 1951; Stavitsky and Arquilla, 1958; Vallo et al, 1964; Merskey et al, 1969). The most important disadvantages of these techniques have been summarised by Herbert (1967a) who stressed the limited duration that the cells could be stored and the considerable variation in the sensitivity of different batches. Preservation of cells is of great importance as a large single batch can be prepared, which will last for at least twelve months, and is particularly important with indirect haemagglutination tests (Ling, 1961; Herbert, 1967a).

Most previous methods for the preservation of red cells in this area of technology have included a formaldehyde fixation procedure (Flick, 1948; Cole and Farrel, 1955; Fullthorpe, 1957; Csizmas, 1960; Ling, 1961; Wide, 1962; Merskey et al, 1966; Herbert, 1967a; Das, 1970a; Haanen et al, 1971a).

Ling (1961) in his comparative study on human cells concluded that pyruvic aldehyde was the best fixative agent. Bing et al (1967) commented that glutaraldehyde was a better reagent than formaldehyde for preservation of sheep cells. A small study was therefore designed to find which aldehyde might be suitable for human red cells destined for the T.R.C.H.I.I. F.D.P. estimation.

Human group O RH negative cells, obtained at routine donor sessions into acid citrate dextrose anticoagulant, were allowed to

settle for 2 - 5 days by gravity, washed three times in 50 volumes of saline and the haematocrit established after centrifugation at 1000 G for 5 minutes. Fixation with formaldehyde was attempted by three different methods : Wide's (1962) modification of the method of Weinbach (1958) and the methods described by Ling (1961) and Herbert (1967b). Pyruvic aldehyde fixation was performed according to Ling (1961) and glutaraldehyde fixation was done according to the standardised method (vide infra).

30 batches of glutaraldehyde, 5 batches of formaldehyde and 4 batches of pyruvic aldehyde were prepared separately on different occasions. The cells were tanned and sensitised the same time under identical conditions (vide infra) and the antifibrinogen titre determined along with negative controls. The results are shown in Table I which shows that glutaraldehyde fixation alone provided consistent batches of cells with no trace of autoagglutination. Although the cells fixed with formaldehyde or pyruvic aldehyde appeared satisfactory on initial testing, a significant proportion developed autoagglutination within a month of storage at $4^{\circ}C$ (Table XV).

These results led to the selection of glutaraldehyde as a preserving agent for human cells and to examine this particular fixation procedure in detail. The basis of this procedure is given below.

Preservation Procedure

Human group O Rhesus negative red cells were obtained from blood collected at routine donor withdrawal into Acid Citrate Dextrose (4 parts to 1part). It was allowed to settle by gravity

Antibody titre and incidence of autoagglutination in batches of human group O cells fixed with a different agent and subsequently tanned and sensitised with human fibrinogen.

No. of batches	Type of Aldehyde	Antibody Titre	No. of batches with Autoagglutination in negative controls
5	Formaldehyde	$\frac{1}{1024 \times 10^3} - \frac{1}{2048 \times 10^3}$	3
4	Pyruvicaldehyde	$\frac{1}{256 \times 10^3} - \frac{1}{512 \times 10^3}$	2
30	Gluteraldehyde	$\frac{1}{1024 \times 10^3} - \frac{1}{2048 \times 10^3}$	0

TABLE I

for 2 - 5 days, the plasma was then removed and the cells concentrated washed three times in 50 volumes of isotonic saline. After the final wash and centrifugation at 1000 g for 5 minutes, the packed cell volume was measured and the cells then transferred to a receptacle immersed in iced water.

A 2% red cell suspension was prepared in 1% glutaraldehyde in phosphate/buffered saline, (see appendix) mixed well and incubated for 30 minutes at 4°C with occasional further mixing. The cells were then washed in 50 volumes of distilled water. After the last wash, a 10% (V/V) suspension was prepared in distilled water containing sodium azide (1 mg/ml) and stored at 4°C.

The glutaraldehyde fixed cells were bright red after fixation and gradually became brown with succeeding washes. They do not lyse in distilled water. They are of normal shape, do not clump and retain their antigenic characteristics. Sensitisation of the cells with a protein antigen was possible even after 2 years of storage at 4°C.

Forty separate batches of glutaraldehyde fixed human red cells were prepared over a period of 2 years and no autoagglutination was apparent in any of these batches.

Although the preservation technique, as described, appeared to be entirely satisfactory it was felt of some importance to study what variables might influence the success of instituting this technique in other laboratories. Accordingly the effects of glutaraldehyde concentration, cell concentration, duration of incubation, temperature, buffer pH and storage were studied.

The effect of relative increase in glutaraldehyde and cell content on the fixation of human red cells.

A 2%, 4%, 6%, 8% and 10% cell suspensions were prepared in 1%, 2%, 3%, 4% and 5% glutaraldehyde, respectively. All mixtures were incubated for 30 minutes at 4°C, washed, tanned and coated in a standard way (vide infra). The results are summarised in Table II and demonstrate that no differences were recorded in the sensitivity of the batches prepared in these different ways.

The effect of incubation time on the fixation of human red cells with glutaraldehyde.

A 2% red cell suspension was prepared in 1% glutaraldehyde and divided into three parts. Each was then incubated for different incubation times, (30, 60 and 120 minutes) at 4°C. The fixed cells were then tanned and coated with fibrinogen (vide infra). The results are shown in Table III and demonstrate that glutaraldehyde fixation is a rapid process, but a prolonged incubation does not appear to be harmful.

The effect of temperature on glutaraldehyde fixation

A 2% red cell suspension was prepared in 1% glutaraldehyde and divided into 3 parts. Each was incubated for 30 minutes at either 4°C, 22°C and 37°C. The fixed cells were washed, tanned and coated under identical conditions. The results in Table IV show no evidence of an effect of these temperature variations on the antibody titre to anti-fibrinogen sera.

The effect of buffer pH on glutaraldehyde fixation

A 2% cell suspension was prepared in 1% glutaraldehyde either

The effect of relative increase in glutaraldehyde and cell content on the fixation of human red cells

ALDEHYDE CONC. (%)	BUFFER pH (P.B.S.)	TEMP ($0^{o}C$)	TIME (MIN)	CELL CONC. (% V/V)	ANTIBODY TITRE	CONTROL
1.0	8.0	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
2.0	8.0	4	30	4	$\frac{1}{2048 \times 10^{3}}$	-ve
3.0	8.0	4	30	6	$\frac{1}{2048 \times 10^{3}}$	-ve
4.0	8.0	4	30	8	$\frac{1}{2048 \times 10^{3}}$	-ve
5.0	8.0	4	30	10	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE II

The effect of varying incubation time on glutaraldehyde fixation

ALDEHYDE CONC. (%)	BUFFER pH (P.B.S.)	TEMP ($0^{o}C$)	TIME (MIN)	CELL CONC. (% V/V)	ANTIBODY TITRE	CONTROL
1.0	P.B.S. 8.0	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	P.B.S. 8.0	4	60	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	P.B.S. 8.0	4	120	2	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE III

The effect of incubation temperature variations on glutaraldehyde fixation

ALDEHYDE CONC. (%)	BUFFER AND pH	TEMP ($0^{o}C$)	TIME (MIN)	CELL CONC. (% V/V)	ANTIBODY TITRE	CONTROL
1.0	P.B.S. 8.0	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	P.B.S. 8.0	22	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	P.B.S. 8.0	37	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE IV

in phosphate buffered saline or citrate phosphate buffer. With variation of pH ranging from 6.4 to 8.0, the cell mixture was incubated for 30 minutes at 4°C and then tanned and coated with fibrinogen under identical conditions (vide infra). The results in Table V show that the variability of buffer and pH had no effect on the antibody titre.

Storage of glutaraldehyde fixed cells

Different batches of human red cells were fixed with 1% glutaraldehyde on different occasions and stored at 4°C in sodium azide. On one day the various batches were removed from the refrigerator, tanned and coated with fibrinogen (vide infra). The antibody titres to antifibrinogen sera were then performed on each batch. The results are summarised in Table VI and show that during a period of storage no deleterious effects were demonstrated.

STUDIES ON THE TREATMENT OF GLUTARALDEHYDE FIXED HUMAN RED CELLS WITH TANNIC ACID

Erythrocytes can be coated with antigens by various means for their use in passive haemagglutination reactions. Although many polysaccarides adsorbed on to red cells after only brief direct contact (Keogh et al, 1948; Neter, 1956), proteins usually require the alteration of the cell surface or the use of coupling agents.

Boyden (1951) discovered that proteins could be adsorbed onto red cells after tannic acid treatment, but the mode of action is not yet clear. Pirofsky et al (1962) observed that tanning not only enhanced the attachment of antigens, but also increased their agglutinability and thus their sensitivity in serological reactions.

The effect of varying buffer pH on glutaraldehyde fixation

ALDEHYDE CONC. (%)	BUFFER AND pH	TEMP ($0^{o}C$)	TIME (MIN)	CELL CONC. (% V/V)	ANTIBODY TITRE	CONTROL
1.0	P.B.S. 8.0	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	P.B.S. 6.4	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	C.P. 8.0	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1.0	C.P. 6.4	4	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE V

ANTIBODY TITRE OF 7 BATCHES OF GLUTARALDEHYDE FIXED CELLS STORED AT VARYING TIMES AND SUBSEQUENTLY TANNED AND SENSITISED UNDER IDENTICAL CONDITIONS.

BATCH NUMBER	DATE OF FIXATION	DATE OF TANNING AND COATING	AGE OF THE FIXED CELLS (MONTHS)	ANTIBODY TITRE OF FRESH CELLS	ANTIBODY TITRE OF SAME CELLS AFTER STORAGE	CONTROL
H1	6.11.69	23.1.72	26	$\frac{1}{2048 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H7	16.1.70	23.1.72	24	$\frac{1}{1024 \times 10^3}$	$\frac{1}{2048 \times 10^3}$	-ve
H16	7.7.70	23.1.72	18	$\frac{1}{1024 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H18	16.6.70	23.1.72	19	$\frac{1}{2048 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H19	22.6.71	23.1.72	7	$\frac{1}{1024 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H17	22.6.71	23.1.72	7	$\frac{1}{2048 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H20	13.6.70	25.8.70	$2\frac{1}{2}$	$\frac{1}{2048 \times 10^3}$	$\frac{1}{2048 \times 10^3}$	-ve

TABLE VI

Herbert (1967a) commented that the chief factor affecting the sensitivity of antigen coated tanned cells was the efficiency of the tanning procedure rather than the quantity of antigen attached to the cells and that this role of tannic acid could be compared to an enzyme such as papain.

The Tanning Procedure

Glutaraldehyde fixed human O Rh negative red cells were washed 3 times in 50 volumes of P.B.S. and the haematocrit was established by centrifugation of the cells at 1000 G for five minutes. The cells were then suspended in P.B.S. as a 2% (V/V) concentration. One volume of this cell suspension was then mixed with 1 volume of fresh 1 in 40,000 tannic acid (see appendix) in P.B.S. and incubated in a water bath at 56°C with occasional mixing. Following incubation the cells were washed three times in 50 volumes of P.B.S.

The tanned fixed cells were immediately coated with human fibrinogen (see appendix) by the method found to give optimal results (vide infra), or the cells were suspended as 10% (V/V) in P.B.S. containing sodium azide (1 mg/ml) and stored at 4°C.

The variables influencing this basic procedure were studied in detail.

The effect of varying tannic acid concentration

Glutaraldehyde fixed cells were washed 3 times in 50 volumes of P.B.S. and a 2% (V/V) cell suspension was made in the same buffer. 1 volume of cell suspension was then mixed with 1 volume of tannic acid in P.B.S. of different concentrations and incubated at 56°C for 30 minutes. The cells were then washed and coated with fibrinogen

(vide infra) and antifibrinogen titres performed.

The results are shown in Table VII and indicate that a maximal antibody titre was obtained with a tannic acid concentration between 1 in 20,000 - 1 in 40,000. The titre gradually decreased with the decreasing tannic acid concentration. No benefit was observed with increasing levels of tannic acid, indeed autoagglutination developed with high concentrations. These observations on human glutaraldehyde treated red cells are comparable to the findings of Wide (1962) and Shioiri (1964) who studied the tannic acid treatment of sheep erythrocytes.

The effect of relative increase of both tannic acid and cell concentration

In order to prepare a large batch of tanned cells which avoids the use of large containers and considerable quantities of washing fluid, this aspect of the possible variables was also investigated.

Glutaraldehyde fixed cells were suspended in P.B.S. in a cell concentration of 2%, 4%, 8% and 10% (V/V), in quadruplicate. Each aliquot was then mixed with tannic acid at the concentration of 1 in 40,000, 1 in 20,000, 1 in 10,000 and 1 in 8,000 respectively and incubated at 56°C for 30 minutes. Following incubation the cells were washed in P.B.S. and coated with human fibrinogen (vide infra). Antifibrinogen titres were then recorded.

The results are shown in Table VIII. The important finding was that of 10% (V/V) suspension of cells was satisfactorily tanned by adding a relatively strong solution of tannic acid (1 in 8,000). This observation was of some practical significance.

The effect of variation in tannic acid concentration

Tannic Acid Conc.	Buffer and pH (P.B.S.)	Temp. (O^oC)	Time (MIN)	Cell Conc. (% V/V)	Antibody Titre	CONTROL
1: 5,000	8.0	56	30	2	$\frac{1}{512 \times 10^3}$	(±)
1: 10,000	8.0	56	30	2	$\frac{1}{1024 \times 10^3}$	-ve
1: 20,000	8.0	56	30	2	$\frac{1}{2048 \times 10^3}$	-ve
1: 40,000	8.0	56	30	2	$\frac{1}{2048 \times 10^3}$	-ve
1: 80,000	8.0	56	30	2	$\frac{1}{1024 \times 10^3}$	-ve
1: 160,000	8.0	56	30	2	$\frac{1}{1024 \times 10^3}$	-ve
1: 320,000	8.0	56	30	2	$\frac{1}{512 \times 10^3}$	-ve

(±) - moderate agglutination

TABLE VII

The effect of relative increase of both cell and tannic acid concentration

Tannic acid Conc.	Buffer and pH (P.B.S.)	Temp. ($0^{o}C$)	Time (Min)	Cell Conc. (%V/V)	Antibody titre	Control
1: 40,000	8	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 20,000	8	56	30	4	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 10,000	8	56	30	8	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 8,000	8	56	30	10	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE VIII

The effect of varying the time of tannic acid treatment

Different authors have used different incubation periods for the tanning reaction. Thus Boyden (1951), George and Vaughan (1962) and Herbert (1967b), considered 10 minutes to be sufficient. Other workers used 30 minutes (Wide, 1962) or 1 hour (Merskey 1966, Das, 1970a), For human cells Ling (1961) allowed 15 minutes, whereas Murakami (1965) allowed 30 minutes, Mertens et al (1969) and Merskey (1969) suggested 1 hour.

Using the standard tanning procedure (vide supra) aliquots of cells were incubated from 15 - 120 minutes, coated with fibrinogen and antifibrinogen titres recorded. The results are summarised in Table IX and demonstrate that the incubated periods studied were not critical.

The effect of different buffer and pH variations on the tanning procedure

Ling (1961) used 0.15M phosphate buffer at pH 6.0 for tanning human erythrocytes, whereas Murakami (1965) and Merskey (1969) used phosphate saline buffer at pH 6.4. Mertens et al (1969) used phosphate citrate buffer at pH 6.4.

Glutaraldehyde fixed cells were washed 3 times in P.B.S. and a 2% cell suspension was made in different buffers at different pH. One volume of a cells suspension was mixed with 1 volume of tannic acid (1 in 40,000) in the same buffer and pH as the cells and the mixture incubated at 56°C for 30 minutes. Following the incubation period the cells were washed in the same buffer and pH and then coated with human fibrinogen and antibody titres performed (vide

The effect of varying the incubation time during tannic acid treatment

Tannic acid Conc.	Buffer and pH (P.B.S.)	Temp. ($0^{o}C$)	Time (Min)	Cell Conc. (% V/V)	Antibody titre	Control
1: 40,000	8	56	15	2	$\frac{1}{2048 \times 10}3$	-ve
1: 40,000	8	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	8	56	45	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	8	56	60	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	8	56	120	2	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE IX

infra). The results are shown in Table X from which it is concluded that cells can be tanned by using either P.B.S. or C.P. buffers at pH 6.4 or 8.0.

The effect of storage on tanned cells

Three batches of tanned cells prepared on different dates were washed in P.B.S. and coated with human fibrinogen under identical conditions by the standard method. Antibody titres of the coated cells were run in parallel and the results are shown in Table XI. It was observed that the age of the cells had no significant influence on the results.

STUDIES ON THE COATING OF GLUTARALDEHYDE FIXED TANNED RED CELLS (HUMAN) WITH FIBRINOGEN

The Sensitisation Procedure

Glutaraldehyde tanned human O Rhesus negative cells were washed 3 times in 50 volumes of C.P. buffer and the haematocrit established by centrifugation at 1000 G for 5 minutes. A 4% cell suspension was then prepared in C.P. buffer and 1 volume was added with 1 volume of a solution of human fibrinogen (10 µg clottable protein/ml) in C.P. buffer, mixed well and incubated for 30 minutes at 37°C. The sensitised cells were then washed 3 times in 50 volumes of C.P. buffer and finally made up as a 10% suspension (V/V) in diluting fluid containing sodium azide (1 mg/ml) and stored at 4°C.

This procedure was adopted as a result of the studies described below.

The effect of different buffer at different pH on the tanning procedure.

TANNIC ACID CONC.	BUFFER AND pH	TEMP. ($0^{o}C$)	TIME (MIN)	CELL CONC. (% V/V)	ANTIBODY TITRE	CONTROL
1: 40,000	P.B.S.8.0	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	P.B.S.6.4	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	C.P.8.0	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve
1: 40,000	C.P.6.4	56	30	2	$\frac{1}{2048 \times 10^{3}}$	-ve

TABLE X

ANTIBODY TITRE OF 3 BATCHES OF TANNED FIXED CELLS-STORED AT VARYING PERIODS AND SUBSEQUENTLY SENSITISED UNDER IDENTIAL CONDITIONS.

BATCH NO.	DATE OF TANNING	DATE OF SENSITISATION	AGE OF THE TANNED CELLS (MONTHS)	ANTIBODY TITRE OF FRESH TANNED CELLS	ANTIBODY TITRE OF AGED TANNED CELLS	CONTROL
H-13	16.3.70	20.1.72	22	$\frac{1}{2048 \times 10^3}$	$\frac{1}{1048 \times 10^3}$	-ve
H-18	13.6.70	10.8.70	2	$\frac{1}{1024 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve
H-19	22.6.70	20.1.71	7	$\frac{1}{2048 \times 10^3}$	$\frac{1}{1024 \times 10^3}$	-ve

TABLE XI

The effect of variations in fibrinogen concentration on the sensitisation procedures

Adsorption of the antigen onto cells depends on the concentration of antigen present in the buffer - cell mixture during the sensitisation procedure. A very small amount, less than 1% of the available antigen, is usually adsorbed onto tanned erythrocytes (Scheibel 1956, Ling et al, 1961). Herbert (1967a) observed that cells could be sensitised satisfactorily using a wide range of antigen concentrations but other authors (Heller et al, 1954, Stavitsky and Ingraham 1964) noted non-specific panagglutination with excess antigen. No studies have been performed on glutaraldehyde fixed human red cells coated with fibrinogen and it seemed appropriate to investigate this problem.

Glutaraldehyde, tanned cells were washed 3 times in 50 volumes of C.P. buffer and a 4% (V/V) suspension prepared in the same buffer. Equal volumes of cell suspension and human fibrinogen (1-100 µg clottable protein/ml in C.P. buffer) were mixed and incubated at 37°C for 30 minutes and then washed in C.P. buffer. The results of varying the fibrinogen concentration between 1 - 100 µg/ml is shown in Table XII. Although a wide range of antigen concentrations can be used, the antibody titre was diminished below the concentration of 10 µg/ml, but a fibrinogen concentration of 50 µg or 100 µg/ml resulted in agglutination of the negative controls. A fibrinogen concentration of 10 µg was therefore used to sensitise the tanned human cells throughout this study.

The effect of varying the type of buffer and pH during sensitisation

Glutaraldehyde tanned cells were washed and a 4% cell suspension

(V/V) prepared in different buffers (P.B.S. and C.P.) at different pH's (6.4 and 8.0). One volume of cells was added to one volume of fibrinogen solution (10 mg clottable protein/ml in corresponding buffers), mixed and incubated for 30 minutes at 37°C. The coated cells were washed 3 times in 50 volumes of the same buffer used for the sensitisation and 10% suspension (V/V) prepared in the same buffer containing bovine albumin 2% (V/V) and sodium azide (1 mg/ml).

The results of antifibrinogen titres are shown in Table XII and demonstrate that the antibody titre was not significantly effected by the type of buffer or pH range used. However, in the haemagglutination inhibition test (T.R.C.H.I.I.) for F.D.P., citrate buffer pH 6.4 tended to give a higher sensitivity, thus C.P. buffer (pH 6.4) was used in the present study not only to obtain higher sensitivity but also to prevent clot formation which has been shown to be an occasional problem when phosphate buffer has been used.

The effect of varying the time of incubation during sensitisation

Glutaraldehyde tanned cells were sensitised in the standard way, but the incubation time was varied from 15 - 120 minutes. The cells were washed in 50 volumes of C.P. buffer. The antifibrinogen titres are shown in Table XII and indicate that satisfactory sensitisation can be obtained in 15 minutes. Any further increase in the incubation time did not influence the sensitivity. In this study a 30 minute period was taken for routine sensitisation.

The effect of varying the incubation temperature during sensitisation.

Glutaraldehyde tanned cells were sensitised as before, with the

EFFECT OF VARIATIONS IN FIBRINOGEN CONCENTRATION, BUFFER AND PH TIME OF INCUBATION, TEMPERATURE AND RELATIVE INCREASE OF FIBRINOGEN AND CELL CONCENTRATION ON THE ANTIBODY TITRE DURING THE SENSITISATION PROCEDURE.

Fibrinogen Conc. (µg/ml)	Buffer and pH	Time (Mins)	Temperature ($0^{o}C$)	Cell Conc. (% V/V)	Antibody titre	Autoagglutinations in controls
			FIBRINOGEN CONCENTRATION			
1	C.P. 6.4	30	37	4	$\frac{1}{512 \times 10^{3}}$	-ve
2	C.P. 6.4	30	37	4	$\frac{1}{512 \times 10^{3}}$	-ve
5	C.P. 6.4	30	37	4	$\frac{1}{1024 \times 10^{3}}$	-ve
10	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve
20	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve
50	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{3}}$	$(\bar{+})$
100	C.P. 6.4	30	37	4		(+)
			BUFFER AND pH			
10	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve
10	C.P. 8	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve
10	P.B.S. 6.4	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve
10	P.B.S. 8	30	37	4	$\frac{1}{2048 \times 10^{3}}$	-ve

$(\bar{+})$ = partial or incomplete agglutination

(+) = complete agglutination

-ve = firm end point with distinct button

Continued ...

TABLE XII

TABLE XII (CONTD.)

TIME OF INCUBATION						
10	C.P. 6.4	15	37	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
10	C.P. 6.4	30	30	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
10	C.P. 6.4	60	37	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
10	C.P. 6.4	120	37	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
TEMPERATURE						
10	C.P. 6.4	30	22	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
10	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
RELATIVE INCREASE OF BOTH CELL AND FIBRINOGEN						
10	C.P. 6.4	30	37	4	$\frac{1}{2048 \times 10^{-3}}$	-ve
20	C.P. 6.4	30	37	8	$\frac{1}{2048 \times 10^{-3}}$	-ve
40	C.P. 6.4	30	37	16	$\frac{1}{2048 \times 10^{-3}}$	-ve

TABLE XII

-ve = firm end point with distinct button

exception that the incubation temperature was varied (22°C or 37°C). The results of the antifibrinogen titres are shown in Table XII. The procedure was not influenced by the range of temperature studied.

The effect of proportional increase in both fibrinogen and cell content during sensitisation

The following experiment was designed to prepare a large volume of sensitised cells, but avoiding the use of large quantities of washing fluid. The tanned cells were washed in C.P. buffer (pH 6.4) and 4 to 16% cell suspension (V/V) prepared in C.P. buffer added to fibrinogen solutions with increasing concentrates (10 - 40 µg of clottable protein/ml) in C.P. buffer. The mixtures were incubated at 37°C for 30 minutes and then washed 3 times in C.P. buffer.

The results of the antifibrinogen titres are shown in Table XII and demonstrate that cells can be sensitised satisfactorily by proportionally increasing the concentration of fibrinogen. Thus a 16% suspension (V/V) of cells was satisfactorily sensitised by 40 µg/ml of fibrinogen. This observation has some practical significance to those engaged in this work, for it reduces a considerable amount of tedious work, and in so doing reduces costs. It has also been shown that further cuts are possible by simply decanting the supernant off at the end of the tanning stage and adding the fibrinogen immediately for sensitisation.

Comparative antifibrinogen titres of human and sheep red cells coated with fibrinogen

Human and sheep cells were fixed with glutaraldehyde and sensitised with or without tanning under identical conditions. The

antifibrinogen titres are shown in Table XIII which demonstrate that the titre of sensitised tanned human cells was 4 - 8 times higher than similarly treated sheep cells and that the directly sensitised human cells (untanned) were 16 - 32 times more than equivalent sheep cells. It was also observed that the tanned sheep cells required less antigen (1 µg/ml) to attain the highest antibody titre. The results also show that the cells autoagglutinate when higher concentrations of fibrinogen (50 - 100 µg/ml) are used for sensitisation of either human or sheep cells.

These results using a fibrinogen/antifibrinogen system are in close agreement with Steele and Coomb's (1964) findings with other antigens. They noted that human cells were 4 times more sensitive than sheep cells.

COMPARATIVE ANTIFIBRINOGEN TITRE OF FIBRINOGEN SENSITISED HUMAN AND SHEEP RED CELLS

A. SENSITISED TANNED FIXED CELLS

FIBRINOGEN CONCENTRATION (µg/ml)	BUFFER AND pH	CONCENTRATION OF CELLS (V/V)	TIME OF INCUBATION (MIN)	TEMPERATURE ($0^{\circ}C$)	HUMAN CELLS		SHEEP CELLS	
					ANTIBODY TITRE x 10^3	CONTROL	ANTIBODY TITRE x 10^3	CONTROL
1	C.P. 6.4	4	30	37	$\frac{1}{512}$	-ve	$\frac{1}{256}$	-ve
2	C.P. 6.4	4	30	37	$\frac{1}{512}$	-ve	$\frac{1}{256}$	-ve
5	C.P. 6.4	4	30	37	$\frac{1}{1024}$	-ve	$\frac{1}{256}$	-ve
10	C.P. 6.4	4	30	37	$\frac{1}{2048}$	-ve	$\frac{1}{256}$	-ve
20	C.P. 6.4	4	30	37	$\frac{1}{2048}$	-ve	$\frac{1}{256}$	-ve
50	C.P. 6.4	4	30	37	$\frac{1}{2048}$	$(\bar{+})$	$\frac{1}{256}$	$(\bar{+})$
100	C.P. 6.4	4	30	37	-	+	-	$(\pm)$

B. SENSITISED UNTANNED FIXED CELLS (DIRECTLY SENSITISED)

30	C.P. 6.4	4	60	37	$\frac{1}{64}$ $\frac{1}{128}$	-ve	$\frac{1}{4}$	-ve

TABLE XIII

+ = complete autoagglutination
$(\pm)$ = moderate autoagglutination
$(\bar{+})$ = slight autoagglutination

STUDIES ON THE PREPARATION OF SENSITISED HUMAN RED CELLS FOR THE F.D.P. ASSAY WITHOUT TANNING.

There is no doubt that one of the problems of the T.R.C.H.I.I. for F.D.P. assays has been the ability in many laboratories to prepare satisfactory reagents, notably the sensitised red cells. The existing procedures involving preservation, tanning and coating are laborious and extremely time consuming and it is probable that much of the difficulties have arisen during this arduous and exacting stage.

Ling (1961) and Bing et al (1967) reported that antigen attachment was possible immediately after fixation; thus omitting a tanning stage. Studies were undertaken to assess whether this reported technical feature could be confirmed and adapted to the attachment of fibrinogen to human red cells for the F.D.P. assay.

Using the simple basic experimental design described in detail in previous chapters the optimal fibrinogen concentration, buffers, pH, incubation time and temperature were delineated. The results of these studies are summarised in Table XIV. They show that it is indeed possible to take human group O Rhesus negative red cells, preserve with glutaraldehyde (1%) and obtain apparently satisfactory coating of fibrinogen to enable satisfactory F.D.P. estimations, without including a tanning stage. In summary, using a preservation procedure described previously, the optimal conditions for direct sensitisation appeared to be as follows: glutaraldehyde fixed cells were washed 3 times in C.P. buffer, 1 volume of a 4% suspension (V/V) in C.P. buffer and 1 volume of human fibrinogen solution (30μg of

INVESTIGATIONS ON THE EFFECT OF VARIATIONS IN FIBRINOGEN CONCENTRATIONS, BUFFER AND pH, TEMPERATURE (DURING FIXATION PROCEDURE) - ON DIRECT SENSITISATION OF FIXED CELLS

FIBRINOGEN CONC. (µg/ml)	TEMP. DURING FIXATION (o^oC)	BUFFER AND pH	TEMP. DURING SENSITISATION (o^oC)	TIME OF INCUBATION (MINS)	CELL CONC. (%V/V)	ANTIBODY TITRE	CONTROL
			FIBRINOGEN CONCENTRATION				
2	37	C.P. 6.4	37	60	4	$\frac{1}{8 \times 10^3}$	-ve
5	37	C.P. 6.4	37	60	4	$\frac{1}{16 \times 10^3}$	-ve
10	37	C.P. 6.4	37	60	4	$\frac{1}{32 \times 10^3}$	-ve
20	37	C.P. 6.4	37	60	4	$\frac{1}{64 \times 10^3}$	-ve
30	37	C.P. 6.4	37	60	4	$\frac{1}{128 \times 10^3}$	-ve
50	37	C.P. 6.4	37	60	4	$\frac{1}{128 \times 10^3}$	-ve
100	37	C.P. 6.4	37	60	4	$\frac{1}{128 \times 10^3}$	-ve
			BUFFER AND pH				
30	37	C.P. 6.4	37	60	4	$\frac{1}{128 \times 10^3}$	-ve
30	37	C.P. 8	37	60	4	$\frac{1}{64 \times 10^3}$	-ve
30	37	P.B.S. 6.4	37	60	4	$\frac{1}{32 \times 10^3}$	-ve
30	37	P.B.S. 8	37	60	4	$\frac{1}{32 \times 10^3}$	-ve

TABLE XIV

CONTD. .....

TABLE XIV (CONTD.)

TEMPERATURE VARIATION DURING FIXATION PROCEDURE

30	37	C.P. 6.4	37	60	4	$\frac{1}{128 \times 10^3}$	-ve
30	4	C.P. 6.4	37	60	4	$\frac{1}{16 \times 10^3}$	-ve
30	37	P.B.S. 8	37	60	4	$\frac{1}{32 \times 10^3}$	-ve
30	4	P.B.S. 8	37	60	4	$\frac{1}{8 \times 10^3}$	-ve

TABLE XIV

clottable protein/ml in the same buffer) were mixed and incubated for 60 minutes at 37°C with occasional further mixing. The cells were then washed 3 times in 50 volumes of C.P. buffer and stored as a 10% cell suspension (V/V) in diluting fluid.

The storage of these cells when compared to glutaraldehyde, tanned, sensitised cells are summarised in Table XV. It can be seen that the antifibrinogen titre of the directly sensitised cells (non-tanned), although identical to the tanned batches when initially tested, after approximately 3 months began to lose their sensitivity. It was concluded that tanning results in more effect binding of the antigen to the red cell membrane and that the time used in this procedure is well spent, as cells obtained retain excellent sensitivity for at least 2 years.

THE F.D.P. HAEMAGGLUTINATION INHIBITION IMMUNOASSAY USING HUMAN RED CELLS.

In the previous studies attempts to develop methods which permit the introduction of sensitised human red cells that may be stored for many months for the T.R.C.H.I.I. for F.D.P. estimation have been described in detail. In terms of antibody titration values and stability, the results of these studies were particularly successful. However, before clinical studies with this new reagent could be considered it was important to observe their behavior in the inhibition immunoassay situation. The apparatus (Microtitre system) and other reagents required, (preparation of test sera, details of antibody titration, patterns of agglutination obtained, controls incorporated into each assay and the calculation of F.D.P.

Antibody titre of fibrinogen sensitised human cells (tanned and untanned) after storage for variable length of time (reciprocal titre).

A. FIBRINOGEN SENSITISED TANNED CELLS FIXED WITH DIFFERENT ALDEHYDE

Batch No.	Initial	DURATION OF STORAGE IN MONTHS 1	2	3	4	5	6	9	12	15	18	21	24	27
						TANNED GLUTARALDEHYDE FIXED CELLS								
H-1	2048T	1024T	2048T	2048T	1024T	1024T	1024T	2048T	2048T	1024T	1024T	1024T	1024T	512T
H-2	2048T	2048T	2048T	2048T	2048T	2048T	1024T	1024T	2048T	1024T	1024T	1024T	512T	1024T
H-3	2048T	2048T	1024T	2048T	1024T	1024T	2048T	2048T	1024T	1024T	1024T	1024T	1024T	512T
H-4	2048T	2048T	1024T	2048T	2048T	1024T	2048T	1024T	1024T	1024T	1024T	1024T	512T	512T
H-5	2048T	2048T	2048T	1024T	2048T	2048T	1024T	2048T	1024T	1024T	1024T	1024T	1024T	1024T
H-6	2048T	2048T	1024T	1024T	2048T	2048T	1024T	2048T	1024T	1024T	1024T	1024T	512T	512T
H-7	2048T	2048T	2048T	1024T	2048T	2048T	1024T	1024T	1024T	1024T	1024T	1024T	1024T	1024T
H-8	2048T	1024T	2048T	2048T	1024T	1024T	2048T	1024T	1024T	1024T	1024T	1024T	512T	512T
HL	2048T	2048T	2048T	1024T	2048T	1024T	2048T	1024T	2048T	1024T	1024T	1024T	1024T	512T
HS	2048T	1024T	2048T	1024T	1024T	2048T	1024T	2048T	1024T	1024T	1024T	1024T	512T	1024T
						TANNED PYRUVIC ALDEHYDE FIXED CELLS								
P.S.	512T	A	A	A	A	A	A							
						TANNED FORMALISED CELLS								
F.S.	2048T	A	A	A	A	A	A							

B. FIBRINOGEN SENSITISED UNTANNED FIXED CELLS

Glutaraldehyde	128T	128T	64T	64T	32T	32T	16T	16T	8T	8T	4T	1T
Pyruvic aldehyde	16T	A	A	A	A	A	A					
Formaldehyde	4T	A	A	A	A	A	A					

A - Agglutination

T - Thousand

TABLE XV

values) have been described in detail by previous workers (Merskey 1969; Das 1970b) and are outlined in the appendix of this thesis. The purpose of this chapter is to summarise the basic principles of the inhibition immunoassay and describe some experiments designed to consider certain important variables which might influence F.D.P. quantitation.

Principle of the test

When diluted antifibrinogen sera is incubated with test samples containing excess fibrinogen or F.D.P. the antisera is neutralised completely. If fibrinogen coated cells are now added, no agglutination occurs. The reaction between antisera and coated cells has therefore been inhibited. If the amount of fibrinogen or F.D.P. is insufficient, then only partial neutralisation of the antisera occurs leaving sufficient to react with the sensitised cells and thus agglutination occurs.

This is the basic principle of all haemagglutination inhibition reaction and by using specific anti-human fibrinogen sera and fibrinogen controls for calibration, the dilution at which the settling pattern changes from inhibition to non-inhibition enables an unknown test sample to be quantitated.

T.R.C.H.I.I. for F.D.P. quantitation

Three standard fibrinogen solutions of varying concentration (58 mg%, 100 mg% and 136 mg% clottable protein) were diluted 100 fold in diluting fluid and then serially diluted in the same way as the test sample. The optimal concentration of antisera for the

assay was determined or calculated from the antibody test obtained from running antisera against sensitised cells (see appendix). In practice the antisera used was approximately 10 times more concentrated than the maximum dilution which produced unequivocal passive agglutination.

Titration of fibrinogen standards and test samples were made serially as 1/2, 1/3, 1/4, 1/6, 1/8 using diluting fluid. Specific attention was paid to such details as meticulous rotating of the micro-dilutor 20 times in each well, to obtain satisfactory mixing. Separate dilutors were used for the 1/2, 1/4, 1/8, 1/16, 1/32 series and the 1/3, 1/6, 1/12, 1/24 and 1/48 series.

One volume (0.025 ml) of antisera, suitably diluted, was now added to all the diluted samples in each well. Mixing was achieved by aggitation of the microtitre plates followed by a period of 4 hours incubation at 22°C (room temperature). One volume (0.025 ml) of a 2.5% suspension of sensitised red cells was then added to every well, thoroughly mixed, incubated at room temperature overnight and the settling patterns recorded the following morning. Positive and negative controls were set up in every assay batch.

Although a considerable number of variables which might effect the T.R.C.H.I.I. for F.D.P. have already been extensively studied (Das 1970b), at least 4 features were regarded as having had less than satisfactory attention or none at all. Accordingly experiments were performed in order to define these variable.

<u>The effect of variations in the concentration of the stabilizing agent in the diluting fluid</u>

In this experiment bovine and human albumin were used as

stabilizing agents and added to citrate buffer at different concentrations (0.25% to 6%). The T.R.C.H.I.I. was performed on standard fibrinogen solutions and on normal as well as pathological sera. The effect on the inhibition is shown in Table XVI which reveals that the inhibition titre increases when the concentration of stabilising agent is increased to 2%. Further increases in protein concentration reverse this trend. Moreover, in pathological sera no inhibition could be detected due to appearance of a prozone phenomenon. At an albumin concentration of 0.25%, auto-agglutination occurred with standard fibrinogen solutions. There was no significant difference between the results obtained from bovine and human albumin. Thus the work reported in this thesis, 2% bovine albumin was incorporated in the diluting fluid as a stabilizer.

A comparison of Phosphate Buffer Saline and Citrate Phosphate Buffer at different pH on the sensitivity of the T.R.C.H.I.I.

Comparative studies were performed to investigate the effect of C.P. and P.B.S. buffers at pH 6.4 or 8.0 on the inhibition titre. The results are shown in Table XVII. There appeared to be little doubt that C.P. buffer at pH 6.4 gave significantly higher inhibition titres as well as better agglutination patterns. Thus C.P. buffer at pH 6.4 was used throughout this study to prepare the diluting fluid.

The effect of temperature on the sensitivity of the T.R.C.H.I.I.

To investigate the effect of incubation temperature on this inhibition titre, 2 microtitre plates containing similar test samples and standard fibrinogen solutions were incubated at different temper-

Effect of concentration of stabilising agent (human or bovine albumin) in diluting fluid in relation to inhibition titre (Reciprocal titre).

	Concentration of Bovine or Human Albumin in diluting fluid								
	0.25%	0.5%	1%	2%	3%	4%	5%	6%	Negative Control
Human fibrinogen (58 mg%)	A	800	1200	1600	1200	400	A	A	A (at 0.25%)
Human fibrinogen (100 mg%)	A	1200	1600	2400	1600	400	A	A	A (at 0.25%)
Normal Serum 1	8	12	16	24	16	12	8	8	O.K.
Normal Serum 2	6	8	12	12	8	8	4	4	O.K.
Normal Serum 3	12	12	16	16	12	12	8	8	O.K.
Pathological Serum 1	6	8	8	8	6(P)	6(P)	4(P)	4(P)	O.K.
Pathological Serum II	16(p)	24(P)	24(P)	24(P)	12(P)	A	A	A	O.K.

TABLE XVI

A = autoagglutination

P = Prozone phenomenon

Effect of pH and buffer on haemagglutination inhibition titre (Reciprocal).

Type of Buffer and its pH	Standard fibrinogen (100 mg%)		Serum sample	
	Inhibition Titre	Quantity at end points (µg/ml)	Inhibition Titre	Quantity of F.D.P. in serum (µg/ml)
C.P. 6.4	1600	0.6	16	9.6
C.P. 8	A	-	12	7.2
P.B.S. 6.4	A	-	4	2.4
P.B.S. 8	A	-	A	-

TABLE XVII

A = autoagglutination

atures (4°C or 22°C) for the same incubation period. The results are shown in Table XVIII. The inhibition titre was higher in those plates which were incubated at 22°C. Moreover, the presence of cold agglutinins in some test sera completely masked the inhibition titre when the microtitre plates were incubated at 4°C. Although the action of the cold agglutinins could be partially reversed by a second incubation at room temperature or 37°C, this was not always successful and accordingly room temperature was routinely adopted for this study.

The effect of varying the interval between addition of antisera and cells on inhibition titre.

The following experiment was designed to study the effect on the inhibition titre by varying the time between addition of antisera and sensitised cells. The test samples including the standard fibrinogen solutions, were diluted in diluting fluid in 8 separate plates. Antisera was added to each plate and the contents mixed. Fibrinogen sensitised cells were then added after fixed incubation periods of 15, 30, 60, 120, 180, 240 minutes and overnight at room temperature. The results are shown in Table XIX. The inhibition titre increased with increasing the incubation period. Thus overnight incubation provided the highest inhibition titre. Moreover, it was noted that to obtain really good unequivocal inhibition patterns in the standard fibrinogen solutions and test samples, the incubation period should be at least 2 hours.

Effect of incubation

temperature on Inhibition Titre (reciprocal).

	Plate Incubated at 4°C	Plate Incubated at 22°C (R.T)
Standard Fibrinogen (58 mg%)	600	800
Standard Fibrinogen (100 mg%)	800	1600
Serum I	A	16
Serum II	12	16

A indicates autoagglutination

TABLE XVIII

Effect of varying an interval between addition of antisera and cells on inhibition titre (Reciprocal).

Type of test samples	Varying interval between addition of antisera and cells							
	0 Min	15 Mins	30 Mins	60 Mins	120 Mins	180 Mins	240 Mins	Overnight
Serum 1	6	12	12	24	24	24	24	32
Serum 2	6	12	12	24	24	24	24	32
Serum 3	6	12	12	24	24	24	24	32
Pooled Control sera	4	8	8	12	24	16	16	24
M.S. 59L	8	16	16	24	24	32	32	48
M.S. 100G	8	16	16	24	24	32	32	48
Standard Fibrinogen (58 mg%)	200	400	400	600	800	800	800	1200
Standard Fibrinogen (100 mg%)	300	600	600	800	1200	1200	1200	1600

TABLE XIX

DISCUSSION AND CONCLUSIONS OF STUDIES ON THE PREPARATION OF PRESERVED, SENSITISED HUMAN RED CELLS FOR THE ASSAY OF F.D.P.

The present investigation was designed to find a substitute for formaldehyde as a preserving agent for human red cells in the T.R.C.H. I.I. for F.D.P., as formaldehyde enhances the tendency to autoagglutinate human red cells. It has been demonstrated that glutaraldehyde is the best fixative for human cells: they react satisfactorily for at least 2 years and the sensitivity of the antigen coated cells remains high. Glutaraldehyde fixed human erythrocytes can be sensitised directly without tannic acid treatment and the sensitivity of such preparations although modest, is satisfactory for routine use. However, compared to glutaraldehyde tanned cells, the storage stability is less satisfactory. This latter finding is similar to those of Bing et al (1967), who studied the attachment of proteins other than fibrinogen to glutaraldehyde fixed sheep cells.

It was interesting to record that glutaraldehyde-tanned cells appeared to have a consistently higher sensitivity than most sheep cell preparations prepared in the same way. This observation confirms the work of Steele and Coombs (1964) who studied antigens other than fibrinogen.

The sensitivity of the cells appeared to depend primarily on the tanning procedure. Thus tanned cells were 32 times more sensitive than untanned. Satisfactory tanning can be achieved over a wide range of tannic acid concentrations (1: 10,000 to 160,000), but concentrations higher than 10,000, of the preparation used in this study increased, caused a high incidence of autoagglutination.

Optimal sensitivity was achieved by using a concentration between 1: 20,000 to 40,000. This aspect of the study supports the findings of Shioiri (1964) who used proteins other than fibrinogen.

Sensitivity and autoagglutination were also found to be influenced by the concentration of fibrinogen used during the coating procedure. The optimal concentration, providing maximal sensitivity and avoiding autoagglutination, was found to be between 10-20 µg/ml. This order of protein concentration is in line with the results of Boyden (1951), Wide (1962), Steffen and Rosak, (1963).

The incubation time for fixation, tanning and coating of human cells did not appear to be critical and in practice this was maintained at 30 minutes. Glutaraldehyde fixation and tanning were performed at pH 8.0 and coating at 6.4.

The cells can be fixed at 4°C, 22°C or 37°C. Although this was done at 4°C for routine use, for direct sensitisation this was best achieved by fixation at 37°C. The temperature for tanning can vary from 22°C to 56°C and the incubation time could be as short as 15 minutes. The sensitisation can be achieved both at 22°C and 37°C. The concentration of fibrinogen required for the coating of tanned cells was 10 µg/ml but 30 µg/ml was needed for direct sensitisation of untanned fixed cells.

These investigations demonstrate that the human cells prepared fulfilled all the criteria required. Thus the following characteristics were observed.

1) Both coated and uncoated cells could be preserved for prolonged periods.
2) Antibody titres were more than a million and thus the sensitivity

in the inhibition reaction was very high.

3) The antibody titre remained almost near to its original titre even after prolonged storage despite repeated washings, which indicate that the antigens were firmly bound to the cells.

4) No interference of non-specific and cold agglutinins were encountered during this study - as the tests were performed and incubated at room temperature (22°C). Thus no absorption procedures were required prior to the assays, with the exception of occasional urine samples.

5) Negative controls always gave well defined settling patterns. Moreover, during storage the inhibition settling pattern gradually improved.

6) The haemagglutination inhibition titre on the same test sera was highly reproducible.

The T.R.C.H.I.I. described proved to be sensitive and very reliable. It was capable of detecting as little as 0.5 µg/ml of fibrinogen. The elimination of an absorption step, which is essential for the sheep cell assay, enabled a trained technician to quantitate as many as 50 serum samples per day.

CHAPTER 7.

STUDIES ON THE AGGLUTINATION OF SHEEP RED CELLS BY HUMAN SERA.

STUDIES ON THE HETEROPHILE HAEMAGGLUTININS TO SHEEP CELLS IN NORMAL HUMAN SERA.

Several authors have reported on the problems of obtaining complete absorption of heterophile agglutinins in normal human sera prior to performing F.D.P. assays when using tanned and sensitised sheep cells (Cash et al, 1969 and Mertens et al, 1969). This problem has not received the attention it deserves as it may well explain some of the anomalous results between different laboratories when studying the same group of patients.

One important source of variability could theoretically be the sheep red cells used for absorption purposes. Accordingly, the following set of experiments were designed to examine the optimal conditions for sheep red cells agglutination by human sera and to investigate the possibility that some sheep red cells might react poorly to heterophile agglutinins and in so doing would prove to be less than ideal for absorption purposes. At the same time it was hoped to isolate a 'high reacting' sheep cell preparation for further studies on the heterophile agglutinins.

MATERIALS

See appendix.

METHODS

Preparation of test sera

Test serum samples were prepared as for F.D.P. estimations: 10 mls of blood were collected in a glass tube containing 0.2 ml of aprotinin (5000 K.I. units/ml). The mixture was incubated at 37°C for four hours and the sera separated after centrifugation at 3400 R.P.M. for 10 minutes. Thrombin was then added to the serum to give a final incubation for 30 minutes at 37°C and centrifuged at 3400 R.P.M. for five minutes. The resultant sera was either tested immediately or stored at -40°C.

The test system

The microtitre system was used throughout, using 0.025 ml volumes. One volume of test sera was titrated in serial doubling dilutions, followed by adding one volume of diluent and finally 1 volume of a 2.5% suspension of sheep red cells. The contents of the microtitre plates were mixed by shaking and then incubated at 4°C overnight (approximately 16 hours). The patterns were read at room temperature.

A system of scoring the agglutination titres arithmetically was used to summarise and analyse the data (Race and Sanger, 1954). A score of 5 was given to each serial dilution, thus the agglutination end points read at 1/1 scored 5, 1/2 = 10, 1/4 = 15, 1/16 = 25, 1/32 = 30.

The intermediate agglutination ($\pm$) scored as 3 and ($\bar{+}$) scored 2.

The absorption procedure

Two volumes of test samples were added to 1 volume of washed

packed red cells, mixed and incubated for 30 minutes at 4°C. After incubation the samples were centrifuged at 3,400 R.P.M. for 5 minutes at 4°C, separated and stored at -40°C.

RESULTS

The effect of incubation temperature

Test sera was diluted (doubling dilutions) in separate plates and the sheep red cells added. Two plates, one for fresh and the other for tanned cells, were incubated overnight at 4°C and the other two plates at room temperature. The results are shown in Table XX which demonstrates that agglutination titres are higher at 4°C incubation than at room temperature.

These results support the conclusion that the heterophile antibody to sheep's cells in human sera reacts like a cold agglutinin and that absorption should be done at 4°C.

The importance of stabilising agent in the diluent

Comparative studies were made of tanned sheep cells in phosphate buffered saline at pH 6.4 or 8.0, in saline and distilled water. The results demonstrated that the cells autoagglutinated in saline, distilled water and buffer (Table XXI). However, this was prevented when a stabilising agent such as horse serum was added to the buffer. No difference was observed between the pH range 6.4 - 8.0.

The effect of Trasylol (Aprotinin) and Thrombin

As it was hoped in future studies (vide infra) to assay the heterophile haemagglutinin titres in the sera, it was important to ascertain whether this could be done on sera obtained for F.D.P. studies, i.e. sera containing Trasylol and

Heterophile agglutination titre in relation to incubation temperature (Receiprocal titres)

	INCUBATION AT 4°C		INCUBATION AT ROOM TEMPERATURE (22°C)	
	Sheep red cells		Sheep red cells	
	Tanned	Fresh	Tanned	Fresh
Serum 24	8	4	0	0
Serum 25	32	16	16	8
Serum 26	32	7	8	2
Serum 27	16	8	4	4
Serum 29	128	8	16	4

TABLE XX

The effect of diluent with or without stabilisation on tanned sheep cells

	DILUENT				
	PBS 6.4	CP Buffer 6.4	Saline	Distilled Water	Diluting Fluid
Tanned Sheep Cells 1	A	A	A	A	(-ve)
Tanned Sheep Cells 11	A	A	A	A	(-ve)
Tanned Sheep Cells 111	A	A	A	A	(-ve)
Tanned Sheep Cells IV	A	A	A	A	(-ve)
Tanned Sheep Cells V	A	A	A	A	(-ve)
Tanned Sheep Cells VI	A	A	A	A	(-ve)

A indicates autoagglutination in negative control

-ve indicates a firm distinct button of cells

TABLE XXI

Thrombin.

12 normal sera were collected either in plain glass tubes or tubes containing 0.1 ml of trasylol. The sera were separated out and then tested for heterophile haemagglutinins against sheep red cells. The results demonstrate that the heterophile agglutination scores, were not significantly effected by these agents (Table XXII).

The effect of cell concentration on the heterophile haemagglutinins titre.

1% and 2.5% cell suspension (V/V) were added to diluted test samples and incubated overnight at $4^{o}C$. The results are shown in Table XXIII. The titres were not significantly altered by using these two concentrations of cells. However, the end-points were difficult to differentiate when a 1% cell suspension was used. Therefore, a 2.5% cell suspension was used for all future tests.

The reproducibility of the test

Thirty coded normal sera were tested fresh and after storage at $-40^{o}C$ for more than 6 months. The results are shown in Table XXIV which shows that the results were reproducible and that storage has no deleterious effects, as there was no statistically significant difference between each set of results ($p > 0.1$).

A comparison of the heterophile agglutination score in human sera to fresh and tannic acid treated sheep red cells

A single batch of cells was selected and tested fresh and after tannic acid treatment against 50 normal sera. The results are shown

The effect of Thrombin and Trasylol on the reactivity of sheep erythrocytes to heterophile haemagglutinins of 12 hormal sera, expressed as scores.

Sample No.	Without Trasylol	With Trasylol	Without Trasylol but added Thrombin	With Trasylol and Thrombin
1.	32	32	33	32
2.	32	28	30	27
3.	35	32	32	30
4.	30	32	30	30
5.	30	30	28	28
6.	28	28	27	25
7.	30	28	27	27
8.	28	28	23	25
9.	27	27	27	27
10.	30	30	28	27
11.	25	28	27	27
12.	30	30	27	27

TABLE XXII

The effect of cell concentration on the heterophile haemagglutination reaction

Serum Sample	1% cell suspension (V/V)		2.5% cell suspension (V/V)	
	Titre	Score	Titre	Score
MS 56 C	1/128	38	1/64	38
NS 10	1/64	35	1/32	30
NS 11	1/64	33	1/32	27
CS	1/64	30	1/64	27

TABLE XXIII

Reproducibility of the Agglutination Test

Sample Number	Heterophile Agglutination scores on day 1	Heterophile Agglutination scores on day 11 (after 6 months storage)
1	32	33
2	33	32
3	27	39
4	28	26
5	30	30
6	30	28
7	27	27
8	25	27
9	25	28
10	22	22
11	15	17
12	22	25
13	22	20
14	20	27
15	30	28
16	33	32
17	30	27
18	28	27
19	30	30
20	28	30
21	28	30
22	30	32
23	28	28
24	30	35
25	30	33
26	30	30
27	30	28
28	30	28
29	25	20
30	32	35

TABLE XXIV

in Table XXV which shows that the tannic acid treated cells gave significantly higher scores when compared with fresh cells ($p < 0.001$).

The variability of sheep red cells in their reaction to haemagglutinins in normal sera.

A panel of 10 batches of sheep cells (4 Merino and 6 Finnish Landrace) were preserved in formaldehyde followed by treatment with tannic acid. (Table XXVI). Sera from 25 healthy volunteers were tested simultaneously against these cells and the results are shown in Table XXVI. There was no significant difference between the mean titre scores of cells S11, S12, S17, S18, S19 and S27 (all Finnish Landrace). There was a highly significant lower reactivity between each member of this group (Finnish Landrace) and S24, S25, S26, ($p < 0.001$) and S28 ($p < 0.002$) (all Merino).

Further analysis of the data revealed that although the red cells from sheep S24, S25 and S26 appeared to be "over all" poor reactors, this did not apply to certain individual reactions. An example of this phenomenon is shown by the performance of serum R against S25 cells which reacted more strongly than S11 cells, whereas S26 cells, which usually mirrored the reactions of S25 cells, failed to agglutinate.

Heterophile agglutination scores before and after tannic acid treatment of same batch of sheep cells

Sample No.	Fresh	Tanned	Sample No.	Fresh	Tanned
1	25	40	26	20	20
2	20	35	27	10	10
3	20	40	28	15	30
4	10	20	29	5	5
5	5	15	30	10	20
6	10	40	31	20	30
7	25	40	32	20	30
8	10	15	33	20	30
9	25	40	34	15	5
10	20	40	35	25	40
11	10	35	36	10	25
12	25	35	37	10	15
13	15	40	38	20	35
14	15	35	39	10	25
15	20	35	40	10	25
16	0	0	41	15	40
17	0	0	42	15	40
18	25	35	43	15	40
19	10	25	44	10	40
20	15	30	45	0	5
21	20	30	46	20	30
22	15	25	47	15	20
23	0	0	48	15	15
24	15	40	49	5	20
25	0	0	50	15	35

TABLE XXV

Haemagglutination titres (expressed as scores) in 25 normal sera tested against a panel of 10 sheep cells

Sheep Identification	Sera Identification																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
S11	20	15	25	20	30	15	5	25	30	30	35	25	20	20	25	15	30	5	20	20	15	20	30	25	30
S12	15	15	25	20	25	15	5	20	20	25	35	20	15	15	15	20	30	10	15	10	15	15	30	20	20
S17	15	15	25	20	30	15	10	25	20	30	40	25	20	20	20	20	25	10	20	15	15	15	30	25	20
S18	25	15	25	25	30	20	10	25	20	30	40	25	20	20	20	15	30	15	20	20	15	20	30	20	30
S19	20	10	20	20	25	15	5	20	20	20	30	15	10	15	10	15	20	10	15	20	15	20	30	25	40
S24	10	5	15	10	15	10	0	10	0	15	20	10	10	10	10	10	15	0	5	0	5	5	20	10	10
S25	15	10	20	15	25	15	5	20	5	15	20	15	10	10	10	10	15	15	10	15	10	15	30	10	10
S26	5	0	5	10	15	0	0	10	0	10	10	10	5	10	5	10	15	0	0	0	0	0	10	10	5
S27	20	15	20	20	25	15	10	20	20	25	40	25	20	20	20	20	25	5	20	15	15	15	30	20	20
S28	15	10	20	15	20	10	0	15	15	15	20	20	15	15	15	15	25	-	-	-	-	-	-	-	-

TABLE XXVI

Absorption studies by using "good" and "poor" sheep cell further substantiated the finding presented above.

DISCUSSION AND CONCLUSIONS

The initial experiments in this section were primarily designed to define the optimal conditions in which the heterophile haemagglutinin in human sera reacts with sheep red cells. In summary, it would appear that the test system can be made specific and reproducible. It is best performed using a 2.5% red cell suspension at $4^{o}C$. An important finding for subsequent clinical studies in this Thesis was the absence of interference by Trasylol and bovine thrombin. This resulted in the preparation of glutaraldehyde fixed, sheep red cells for future studies. They proved to be stable and highly reproducible and therefore, an ideal tool for long-term investigations (vide infra).

An extensive search of the literature has failed to reveal previous reports on differences in reactivity between sheep cells to haemagglutinin(s) in normal human serum, although this phenomenon has been observed in sera from patients with infectious mononucleosis (I.M.) (Zarafonetis and Oster, 1950). Moreover, Cox and Vermillion (1956) suggested that the problem was sufficiently important to warrant the preservation of sheep red cells in order to obtain standardised techniques for the serological diagnosis of I.M.

Although the 2HK, AA Finnish Landrace sheep gave titres significantly higher than the others, particularly the 2LK, AB Merinos, and it is possible that the variation of agglutination titres may be partly explained by inherent genetic differences in the cell membrane, the sample size was not sufficient to justify a distinction being made between the effects of breed and blood group. It is also likely that an additional heterogenicity was created by variability in the strength of binding of different populations of anti-sheep cell haemagglutinins.

The practical significance of these results is of particular interest for it is suggested that sheep cells with a known low reactivity to anti-sheep cell haemagglutinins should be selected as the ideal inert carriers of antigen or antibody in all types of haemagglutination immunoassays. In doing so it may prove possible to abandon the time-consumming exercise of absorption prior to carrying out the immunoassays. In those circumstances where this does not prove possible, absorption should be done with known highly reacting cells which must be done at $4^{o}C$. The data also suggests that the conflicting reports of the rise in titre of anti-sheep cell haemagglutinins associated with allograft rejection (Rapaport et al, 1968; O'Kane et al, 1969) may be explained on the differences in reactivity of the sheep cells used in the different centres. It seems likely that the introduction of a panel of sheep cells in future studies would be more revealing.

As a direct result of these methodological studies a stuitable sheep cell preparation was obtained and the optimal conditions for the reaction ascertained for the subsequent clinical investigations.

CHAPTER 8.

STUDIES ON A DIRECT LATEX AGGLUTINATION TECHNIQUE FOR F.D.P. QUANTITATION.

INTRODUCTION

The prime motivation for the F.D.P. technical developments, already described in detail, was towards a linear study in renal disease. The design of this study necessitated the assay of a large number of samples, and using the T.R.C.H.I.I. this would prove to be a particularly laborious exercise.

In 1971 we were given the opportunity of carrying out a series of studies on a new approach to the problem - a direct latex agglutination test, developed at the Wellcome Research Laboratories. This method had several attractions: it was rapid, and considered to be sensitive to fragment E, which did not apply to the existing latex technique known as the Fi-test (Thomas et al, 1970). This latter feature is believed to be due to a coating of antisera directed against the anti D and E, rather than anti-fibrinogen.

In view of the potential usefulness of this reagent, known as the Thrombo-Wellcotest, both for this project and general clinical application its performance has been tested against the T.R.C.H.I.I. on artificial digests of fibrinogen and clinical samples of urine and serum.

MATERIALS AND METHODS

Serum and urine test samples were prepared as described previously (Clarkson et al, 1970 and Das et al, 1967). All concentrated urine samples were centrifuged at 3,400 R.P.M. for 10 minutes at 4°C in a 6L M.S.E. Mistral centrifuge prior to assay. The T.R.C.H.I.I. was performed using the modification of Das (1970b) of the Merskey technique (1966). The Thrombo-Wellcotest reagent was kindly supplied by Wellcome Reagents Limited, Bechenham, Kent, England, as was a suspension of latex particles coated with anti F.D.P. and normal rabbit IgG. Test samples and standard (human fibrinogen; Kabi Pharmaceuticals) were doubly diluted in a glycine buffer (0.1 M in 1% NaCl containing 0.1% sodium azide at pH 8.2. Two drops at a total of 0.05, of test material or standard, were placed into a marked ring on a clean glass slide. Two drops of the latex suspension were then added, mixed using a glass rod and incubated at room temperature for 2 minutes. Macroscopic agglutination was visualised against a dark back-ground.

RESULTS

Sensitivity to fibrinogen, Fragments D and E

The direct latex test was shown to be sensitive to as little as

4 µg/ml of fibrinogen. Specific products (kindly supplied by Doctor V. Marder, Temple University, Philadelphia, U.S.A.) revealed a sensitivity to fragment D of 4 µg/ml and fragment E of 0.5 µg/ml.

Reproducibility

Standard fibrinogen solutions, at different concentrations, citrated plasma, pooled sera and test samples of both serum and urine were tested on different occasions. The results are shown in Table XXVII which demonstrates satisfactory reproducibility.

Precision and sensitivity of the test in relation to incubation time

The precision as well as the sensitivity of the test could be imporved by increasing the incubation time from the usual 2 to 15 minutes. This is summarised in Table XXVIII.

The precision was increased by using two drops instead of 1 drop as recommended. This simple adaption prevented false agglutination reactions in icteric and haemolysed sera. The results are shown in Table XXIX.

Reproducibility of the test

Test samples	(Quantity of F.D.P. (μg/ml)	
	Day - 1	Day - 2
Serum 3781	64 μg/ml	64 μg/ml
Serum 3756	64 μg/ml	64 μg/ml
Serum 3806	64 μg/ml	64 μg/ml
Serum 3818	8 μg/ml	12 μg/ml
Serum 3856	12 μg/ml	16 μg/ml
Control pooled serum	8 μg/ml	8 μg/ml
Plasma	4096 μg/ml	4096 μg/ml
Urine 7508	512 μg/ml	512 μg/ml
Urine 7525	1024 μg/ml	1024 μg/ml
Urine 7569	128 μg/ml	128 μg/ml
Urine 7608	8 μg/ml	8 μg/ml
Urine 9047	256 μg/ml	256 μg/ml
Urine 1932	16 μg/ml	12 μg/ml
Urine 9125	64 μg/ml	64 μg/ml
Urine 9130	16 μg/ml	16 μg/ml
H.F. 1.25 mg/ml (W/V)	512 μg/ml	512 μg/ml
H.F. 2.5 mg/ml (W/V)	1024 μg/ml	1024 μg/ml
H.F. 5.0 mg/ml (W/V)	2048 μg/ml	2048 μg/ml

TABLE XXVII

Sensitivity and precision of the test in relation to incubation time

Time required to enhance macroscopical Agglutination (Incubation Time)

Quantity of clott -able protein (µg/ml)	1000	350	250	150	125	90	60	40	30	20	10	5	1	0.5
H.F. 137.0mg/ 100 ml	4'	150"				75"				120"		8'		
H.F. 100mg/100 ml	4'		90"				75"				4'	7'		
H.F. 58mg/100 ml		140"		75"				105"			$4\frac{1}{2}$'			
Plasma	5'		90"				60"			$2\frac{1}{2}$'	5'	7'		
Dig Fibrinogen 300 mg/100 ml (0 min. incubation)							70"			100"		8'		
Dig Fibrinogen (66 hours incuba- tion) 300 mg/100 ml							3'			$3\frac{1}{2}$'		15'		
D. Fragment (100mg/100 ml)								15'		6'		15'		
E. Fragment (100mg/100 ml)								15'		6'		10'		15'
Cont. Fibrinogen (300mg/100 ml)							60"			140"		8'		
Urine					50"			8"			3'	15'		
Serum						60"			90"			7'		

TABLE XXVIII

Precision of the test increased by two drops method

Number of test samples	Number of no false reaction by 1 drop method	Number of false reaction by 1 drop method - which was overcome by two drops method
Normal Sera 100	95	* 5

* indicates 3 test samples were haemolysed and two test samples were icteric.

TABLE XXIX

A comparison of the latex and T.R.C.H.I.I. reactivities during in vitro fibrinogenolysis

Streptokinase (1000 units/ml), plasminogen (0.25 casinolytic units/ml) and purified human fibrinogen (10 mg/ml) were prepared in tris buffer. The mixture incubated at 37°C and at different times, from zero to 66 hours, 1 ml aliquots of the digest were removed and the enzymatic reaction arrested by adding 0.1 ml of Aprotinin (5000 U/ml) and the thrombin time (Thomson, 1970), a fibrinogen estimation (Ellis and Stransky, 1961), T.R.C.H.I.I. and Latex test performed.

The results are summarised in Fig. 5.

The apparent higher sensitivity of the Latex test to later digests, compared to the T.R.C.H.I.I., was thought to be related to the use of antisera in the T.R.C.H.I.I. directed against whole fibrinogen, thus being relatively insensitive to anti-E. This conclusion was supported by the known high sensitivity to the E fragment of the Latex test and the observation that when a specific anti-E serum was used in the T.R.C.H.I.I. then continued sensitivity was retained throughout the period of digestion (Fig. 6).

Comparison of the T.R.C.H.I.I. and Latex Test on Clinical Material

A plot of the results from both techniques on 144 sera and 37 urines is shown in Fig. 7. There was a very satisfactory correlation between the two methods ($r = 0.8942$: $p < 0.001$), despite the use of antisera to whole fibrinogen in the T.R.C.H.I.I.

Non-specific latex particle agglutination

Less than 1% of 120 sera obtained from healthy blood donors

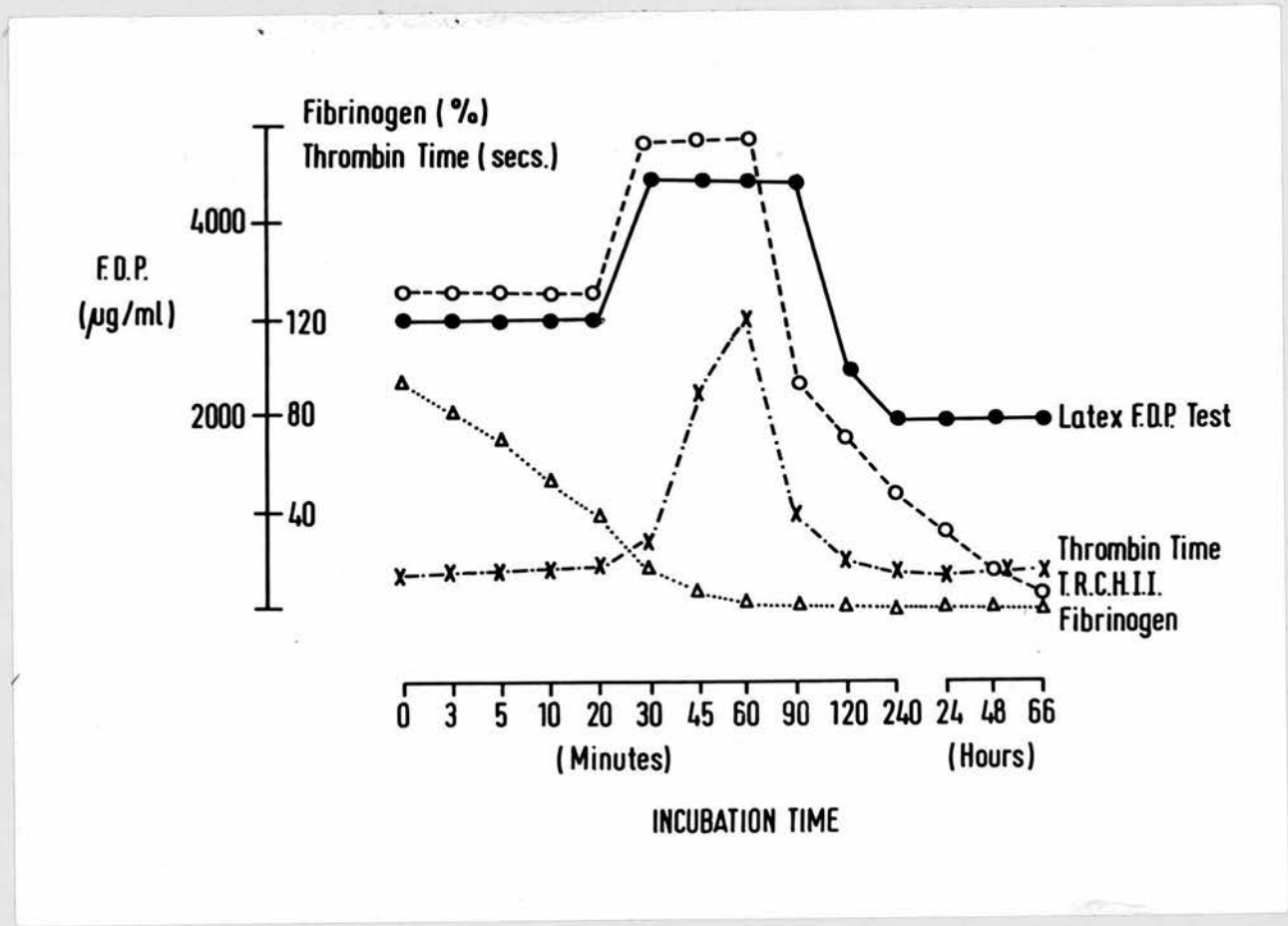

Fig. 5 F.D.P. quantitation using the Latex Test, T.R.C.H.I.I., thrombin time and fibrinogen estimations on serial aliquots of an in vitro human fibrinogen plasmin digest.

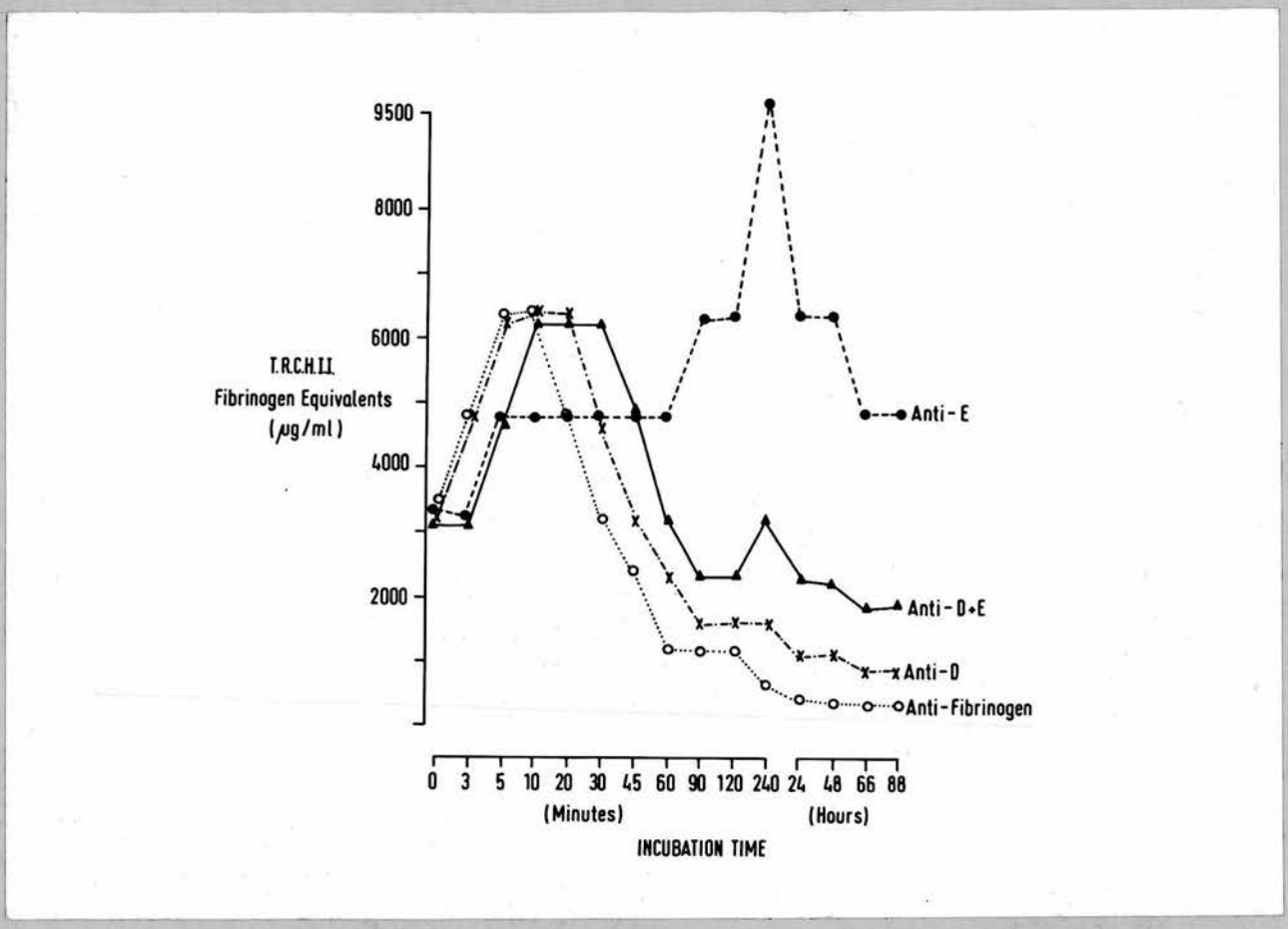

Fig. 6 Results of the T.R.C.H.I.I. on in vitro human fibrinogen digests using antisera prepared to whole human fibrinogen, fragment D and fragment E.

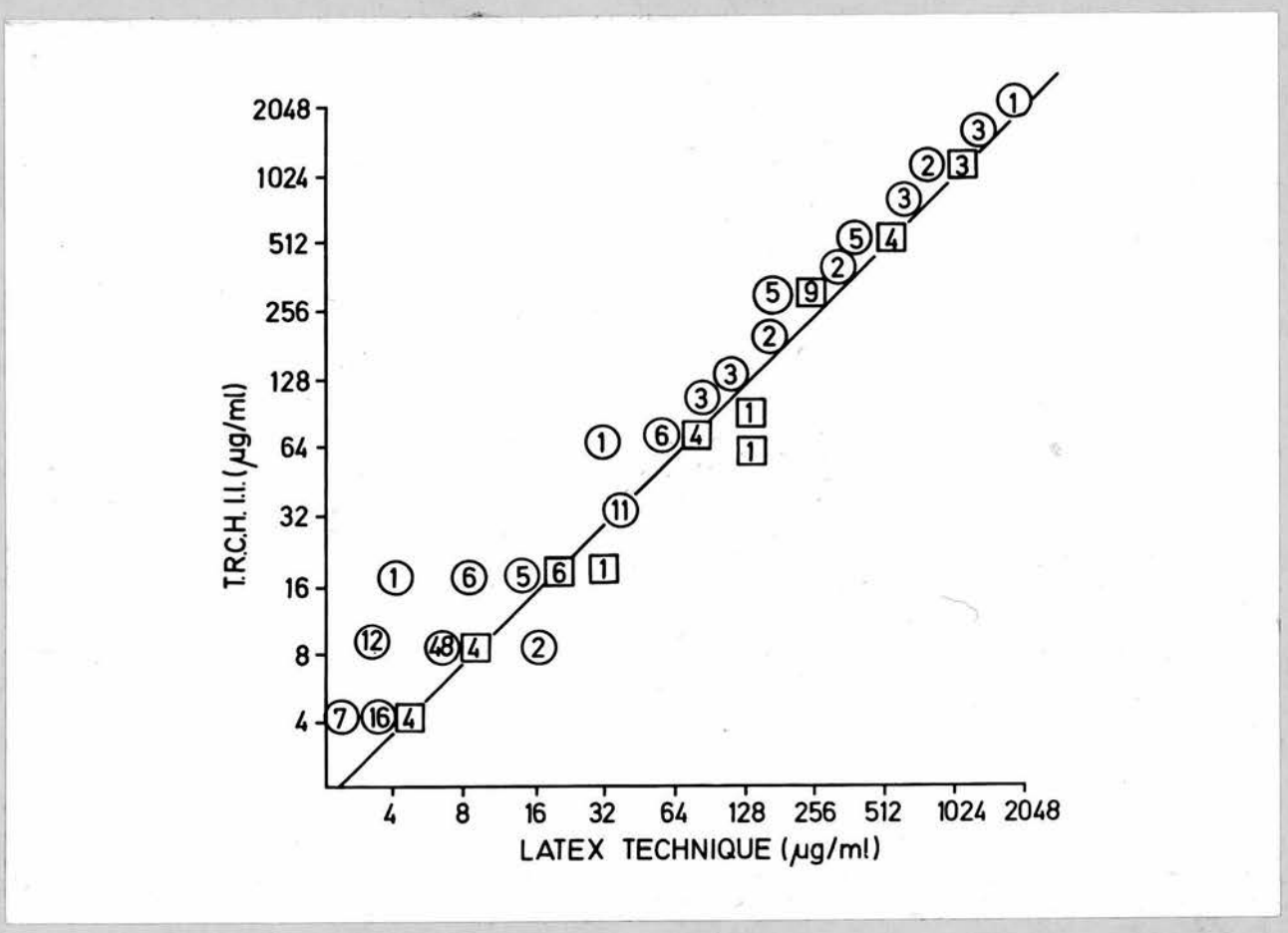

Fig. 7 A correlation between the Latex test and T.R.C.H.I.I. on the F.D.P. values in sera (circles) and urines (squares), ($r = 0.894$, $p < 0.001$).

agglutinated the latex particles coated with normal rabbit IgG. However, in patients with rheumatoid arthritis this rose to 25%: the highest titre recorded being 1/8. In urine specimens, whether from healthy subjects or patients with glomerulonephritis, this rose to 50% in fresh specimens but could be reduced to 25% after freezing, thawing and centrifugation. The highest titre recorded was 1/64. Complete removal of this non-specific latex agglutinating material was possible if 1 volume of urine was incubated with 1 volume of normal rabbit IgG coated latex for 30 minutes at $4^{\circ}C$.

DISCUSSION AND CONCLUSIONS

Latex agglutination tests have been used since 1956 when Singer and Plotz introduced latex particles as carriers for gamma globulin. It has been used most frequently in rheumatology and pregnancy testing. Hirsch et al, 1965 used the Fi-test (antifibrinogen serum coated latex made by Hyland Laboratories) for the estimation of plasma fibrinogen. Since then many others have used the Fi-test for the detection of F.D.P. in serum but with less than satisfactory results (Merskey, et al, 1966; Castelan et al, 1968; Melligar, 1970; Thomas et al, 1970; Marder et al, 1971). Allington 1971 introduced fibrinogen coated latex for estimation of serum F.D.P. using an agglutination inhibition test technique similar to the T.R.C.H.I.I. The Fi-test has the disadvantages of low sensitivity which has varied from 10 - 20 μg/ml and cannot detect late products (Castelan et al, 1968; Thomas et al, 1970; Marder et al, 1971). Allingtons technique, although more sensitive and reliable than the Fi-test, is technically cumbersome (Allington, 1971) and has proved to be

insensitive to the E fragment (Marder et al, 1971).

From the results of this study there seems to be little doubt that the latex F.D.P. test, developed at the Wellcome Research Laboratories, has many attractions for those concerned with the semiquantitation of F.D.P. in serum. In vitro studies above reported have clearly demonstrated that the sensitivity to all digested fragments is good and in terms of fragment E more sensitive than the T.R.C.H.I.I. using whole anti-fibrinogen sera. From a clinical point of view only 20 minutes are required to produce a results, and it must therefore represent a satisfactory technique. The presence of non-specific latex agglutination substances in sera is unlikely to be of significance as it has not been recorded above a titre of 1/8 which corresponds to an F.D.P. of 32 μg/ml. The close correlation between the T.R.C.H.I.I. and latex F.D.P. test on individual serum samples was also encouraging and indicates that laboratories can convert to this assay readily. Although ideally suited as a 'one-off' rapid bedside technique the method is somewhat laborious as there is no readily available and suitable automatic dilution equipment at the moment, and it seems likely that the T.R.C.H.I.I. will continue to be used on those research studies which involve the assay of a large number of specimens. This conclusion was incorporated in the work described in this Thesis.

Merskey et al, (1966) recorded the presence of a significant number of apparent false positive reactions in normal sera when using the Fi-test. This phenomenon was noted in the present studies but it was found that the incidence could be greatly reduced by using

glycine buffer as a diluent in place of normal saline. The mechanism of this reaction is unknown, but by incorporating the glycine buffer the incidence of non-specific latex agglutination in normal sera was reduced to 1%. In practice it seems reasonable to institute a latex control test using normal rabbit IgG coated latex in those sera reading between 20 - 40 μg/ml, as this was the maximum non-specific titre recorded.

The position with regard to the latex F.D.P. assay for urine appears to be quite unlike the serum. Because the latex test has an overall, slightly lower sensitivity than the T.R.C.H.I.I. the need to concentrate the urine specimens before assay is imperative. This procedure greatly increases the non-specific latex agglutination problem so that almost 50% cannot be assayed. Freezing, thawing and centrifugation reduced this to approximately 25% only.

Although the residual non-specific substance(s) could be absorped using normal rabbit IgG coated latex this extra procedure made the whole process extremely laborious. Thus it would appear that for urine F.D.P. assays, the Latex Test is not ideal and in view of the importance of running large batches of assays on individual patients (Clarkson et al, 1971) it is felt that the latex approach is unlikely to replace the T.R.C.H.I.I. for urine assays.

It is of interest to record that the non-specific latex agglutinating substance(s) in sera and urine did not appear to be sensitive to mercaptoethanol treatment and their was no correlation between the non-specific latex agglutinating titres and the sheep red cell heterophile antibody titre in either serum or urine.

SECTION III

STUDIES ON THE URINARY HETEROPHILE HAEMAGGLUTININ AND F.D.P. CONTENT IN GLOMERULONEPHRITIS AND HOMOTRANSPLANTATION.

CHAPTER 9.

INTRODUCTION

At the present time perhaps the most fruitful area in clinical medicine, in which the measurement of F.D.P. appears to offer assistance in the management of individual patients lies in general nephrology and renal transplantation, and in urine rather than serum. Abnormal urine F.D.P. excretion has been shown to arise in glomerulonephritis and during the rejection of renal homografts (Clarkson et al, 1970; Haanen et al, 1971 and Bouma et al, 1971). In proliferative forms of glomerulonephritis urine F.D.P. excretion appears to correlate closely with disease activity (Clarkson et al, 1971), and particularly interesting has been the response to certain therapeutic manoeuvres, both in the rejecting renal homograft (Clarkson et al, 1970) and active proliferative glomerulonephritis (Vermylen et al, 1971 and Clarkson et al, 1972). These events are likely to be immunological in origin (vide supra).

One of the outstanding features of all these studies has been the relative insensitivity of serum F.D.P. to marked activity within the kidney tissue. This in part may be due to reduced glomerular perfusion during the active phase of the disease processes, the fact that much of the intra-renal fibrin is likely to be extravascularly situated (Davison et al, 1973) and due to a dilution factor, as the renal mass accounts for only 25% of the total cardiac output. In

considering how to apply the assay of heterophile haemagglutinin as a marker of non-specific immunological damage in the kidney, it was concluded that its application would be best directed in the first instance towards urine rather than serum. Moreover, the non-invasive nature of a urine analysis has considerable practical attractions, so that patients can be under continued surveillance with the minimum of discomfort and inconvenience.

The following account describes a series of preliminary studies in which an attempt is made to ascertain whether heterophile haemagglutinins are excreted in healthy subjects, those with renal homografts and in glomerulonephritis, and whether this correlates with the urine F.D.P. excretion in these patients. Efforts are also made to ascertain the mechanisms of these phenomena and to delineate their future potential in the care of patients.

CHAPTER 10.

MATERIALS AND METHODS

The normal urines (100, aged 19 - 58 years) were obtained from laboratory personnel and the author's colleagues and friends. They were not 24 hour-collects, but obtained at random during the working day.

The patient groups were those attending the Nuffield Transplant Unit, Western General Hospital, Edinburgh under the care of Professor Sir Michael Woodruff and Mr. B. Nolan; the Medical Renal Unit, Royal Infirmary, Edinburgh under the care of Doctor J.S. Robson and Doctor Anne Lambie; the Paediatric Renal Unit, Royal Hospital for Sick Children under the care of Doctor William Uttley and several other General Medical Units in the S-E Region of Scotland. Aliquots from 24 hour collects were obtained during in-patient periods, but while at home patients sent early morning urine specimens to the laboratory, through the postal services. No proteolytic inhibitors were used during sample collection as this has been shown to be unnecessary (Clarkson et al, 1971).

The diagnoses in the patients with glomerulonephritis was established by light, electron and fluorescent microscopy in the University Department of Pathology under the direction of Doctor Mary K. Macdonald. The urinary total protein estimations were performed in the University Department of Clinical Chemistry, under the direction of Professor G. Whitby. The biuret method used as

described by Hiller et al (1948). The IgG, IgM and C_3 content of the concentrated urines were quantitated by the Mancini technique (1965) using commercially obtained plates (Hyland). Using standard sera the limit of sensitivity of this technique was, C_3 - 3 mg%, IgM - 2.5 mg% and IgG - 5 mg%.

The activity of the disease in each patient was assessed in the light of information available at the time of the urinary study. Cases were considered to be in an active phase if any one or more of the following clinical pathological features were present: decreasing creatinine clearances, progressive rise in the concentration of serum creatinine, proteinuria in excess of 1 G per 24 hours, red blood cell casts in the urine, focal or diffuse proliferation of endothelial and mesangial cells, and intraglomerular fibrin. The disease was considered inactive or resolving if none of these clinical features were present or if histology showed only foci of glomerular sclerosis with or without capsular adhesions.

All urine specimens were first dialysed against tap water and concentrated overnight, using polyethylene glycol (10 - 20 times), as previously described (Clarkson et al, 1970). They were then stored in plastic tubes at -36°C until assayed. All specimens were centrifuged at 3,400 R.P.M. for 30 minutes after thawing prior to assay. In those patients in which serial samples were studied, all were thawed and assayed at the same time in order to maintain identical assay conditions. The assay of urinary F.D.P. and heterophile haemagglutinins (using high reacting sheep cells) were run independently and blindly on coded samples as described in previous chapters and appendix. No prior laboratory or clinical information was available to the person responsible for the assays. The

heterophile haemagglutinin content was expressed in arbitrary units (vide supra).

CHAPTER 11.

RESULTS

URINE F.D.P. AND HETEROPHILE (SHEEP) HAEMAGGLUTININ CONTENT IN HEALTHY VOLUNTEERS

In the 100 normal subjects studied the concentration of urinary F.D.P. never exceeded 0.25 μg/ml which is in agreement with the findings of Clarkson et al (1970; 1971). On no occasion was heterophile antibody to sheep cells detected in normal concentrated urine.

URINE F.D.P. AND HETEROPHILE (SHEEP) HAEMAGGLUTININ EXCRETION IN GLOMERULONEPHRITIS AND HOMOTRANSPLANTATION

A series of 60 selected urines were obtained from the deep freeze and assayed 'blindly' in order to ascertain the degree of correlation between their F.D.P. and heterophile antibody contents. The results are as shown in Fig. 8. Although there was a highly significant correlation ($p < 0.001$) the 'r-value' was less than 0.5 and it was assumed to be due to the presence of high F.D.P. and low heterophile antibody content in some urines and vice versa. This was confirmed when individual patients' urines were studied over a prolonged period, an example of which is shown in Fig. 9. Nevertheless, there was sufficient evidence to suggest that although the results from individual samples might be misleading, when patients were studied serially over a period of time then active proliferative glomerulonephritis was associated with the presence of intermittent

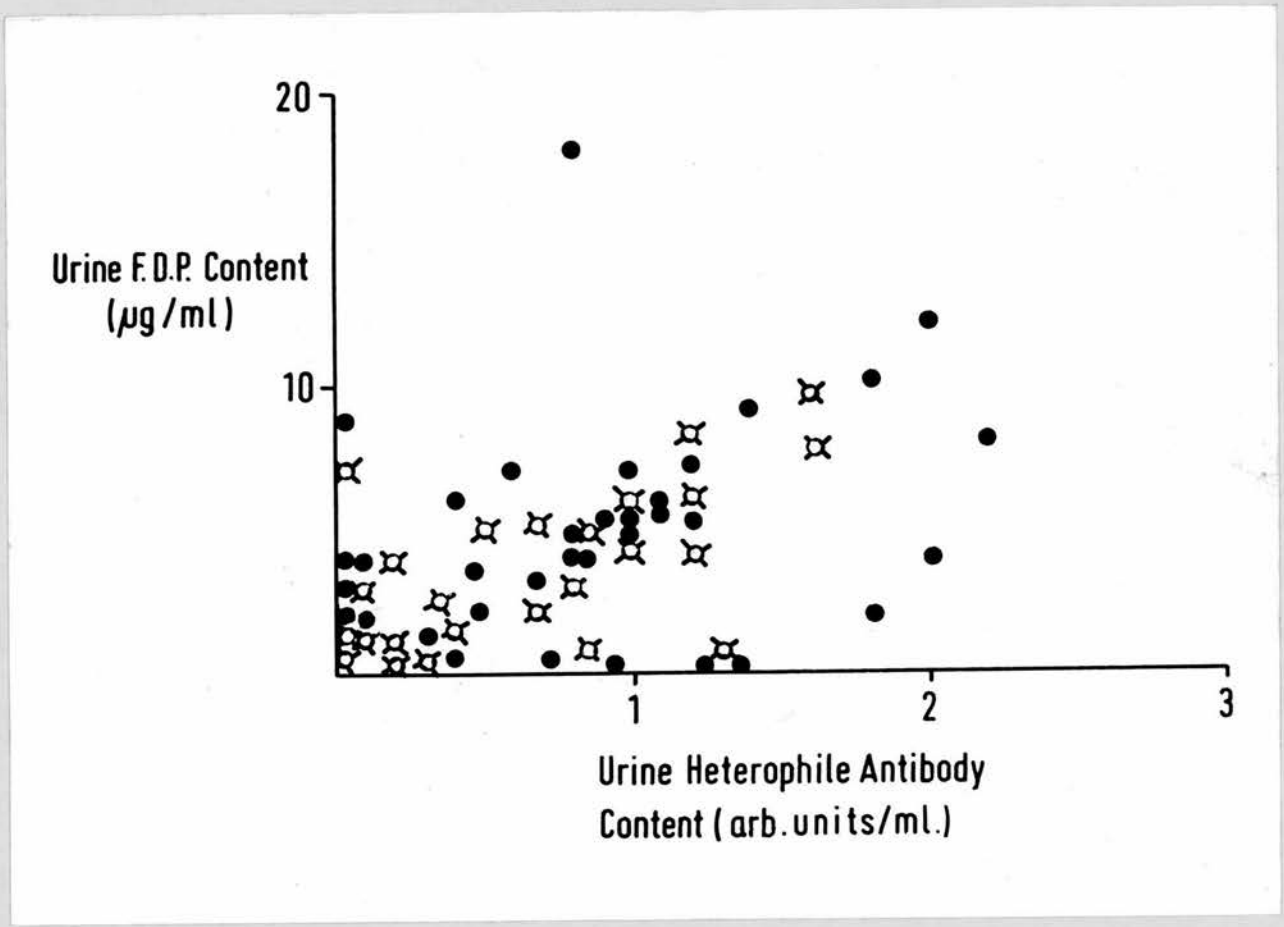

Fig. 8 A correlation between F.D.P. and heterophile (sheep) antibody (haemagglutinin) content in 60 selected urines obtained from patients with proliferative glomerulonephrits (●) and following renal homotransplantation (⊠) [$r = 0.4891$, $p < 0.001$].

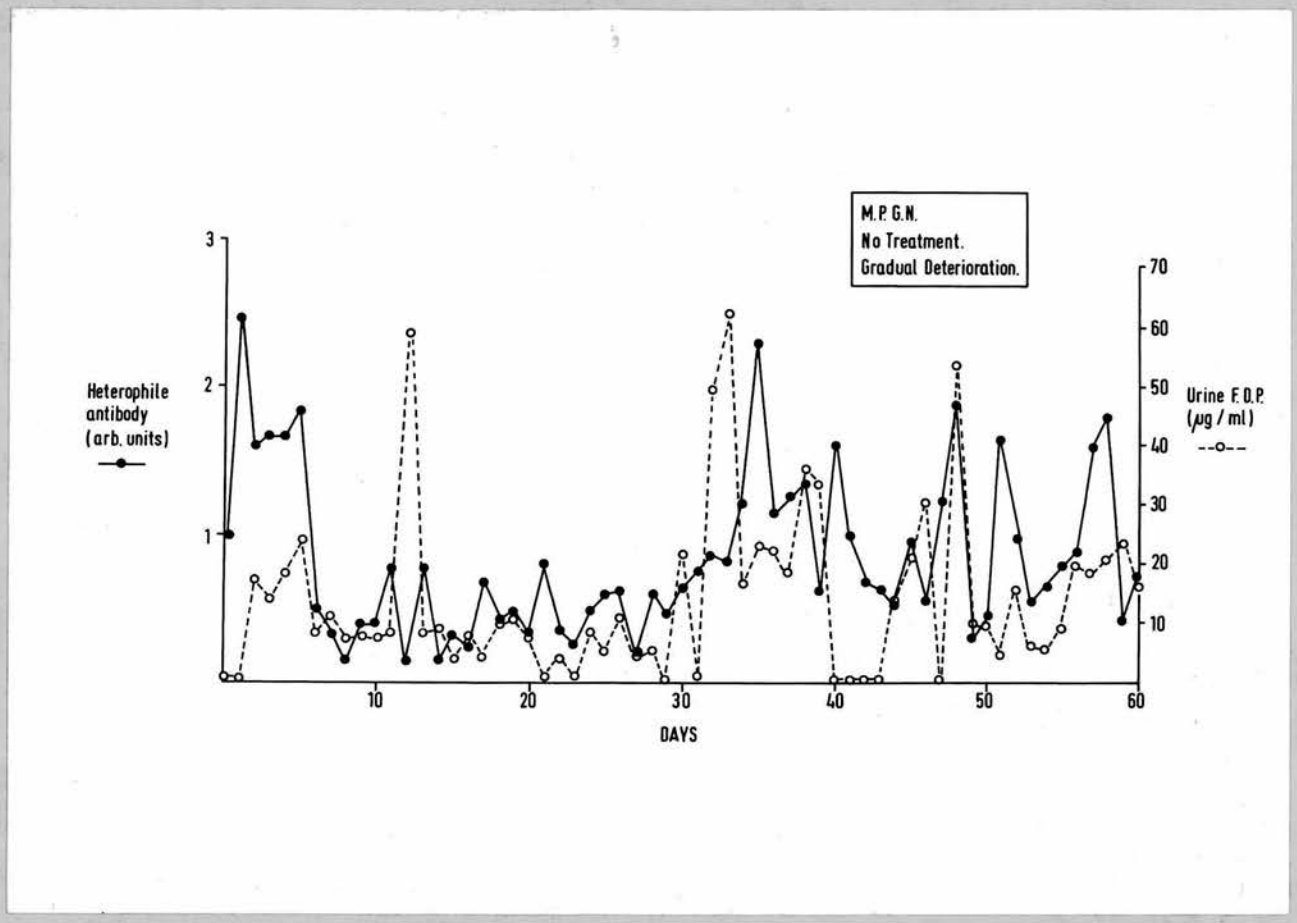

Fig. 9 The pattern of urinary F.D.P. and heterophile (sheep) antibody from a patient with proliferative glomerulonephritis. (Note Some urine specimens contain low F.D.P. and high heterophile antibody and vice versa).

heterophile (sheep) haemagglutinin excretion; a phenomenon never observed in normal controls. Moreover, this was paralleled by the intermittent appearance of abnormal urine F.D.P. excretion in those patients.

These pattern of changes were sharply contrasted in those patients with minimal lesion glomerulonephritis. In this condition (six cases studied serially for 10 - 22 days) there was less than 2 μg/ml of urine F.D.P., which confirms the findings of Clarkson et al (1971), but abundant evidence of heterophile (sheep) haemagglutinin excretion. Moreover, this returned to normal as the patient recovered during steroid therapy (Fig. 10).

The data obtained from patients with active proliferative glomerulonephritis appeared to parallel the findings in patients undergoing acute or subacute renal homograft rejection. Ten transplant patients were studied serially and all clinical episodes of graft rejection were associated with the expected elevation in urine F.D.P. excretion but also an appearance of urinary heterophile haemagglutinin (sheep). Fig 11 shows an example in which the daily events occurring 42 days following renal transplantation are recorded. By the 22nd day there was evidence of rejection, as the urine volume began to fall and serum creatinine was rising. Prior to this, however, there was a rapid rise in the titre of heterophile antibody and an associated elevation in urine F.D.P. Of equal significance was the fall in these latter parameters as the rejection episode was successfully treated, so that by the 31st day the urine volume had returned to satisfactory levels and the serum creatinine was within the normal range.

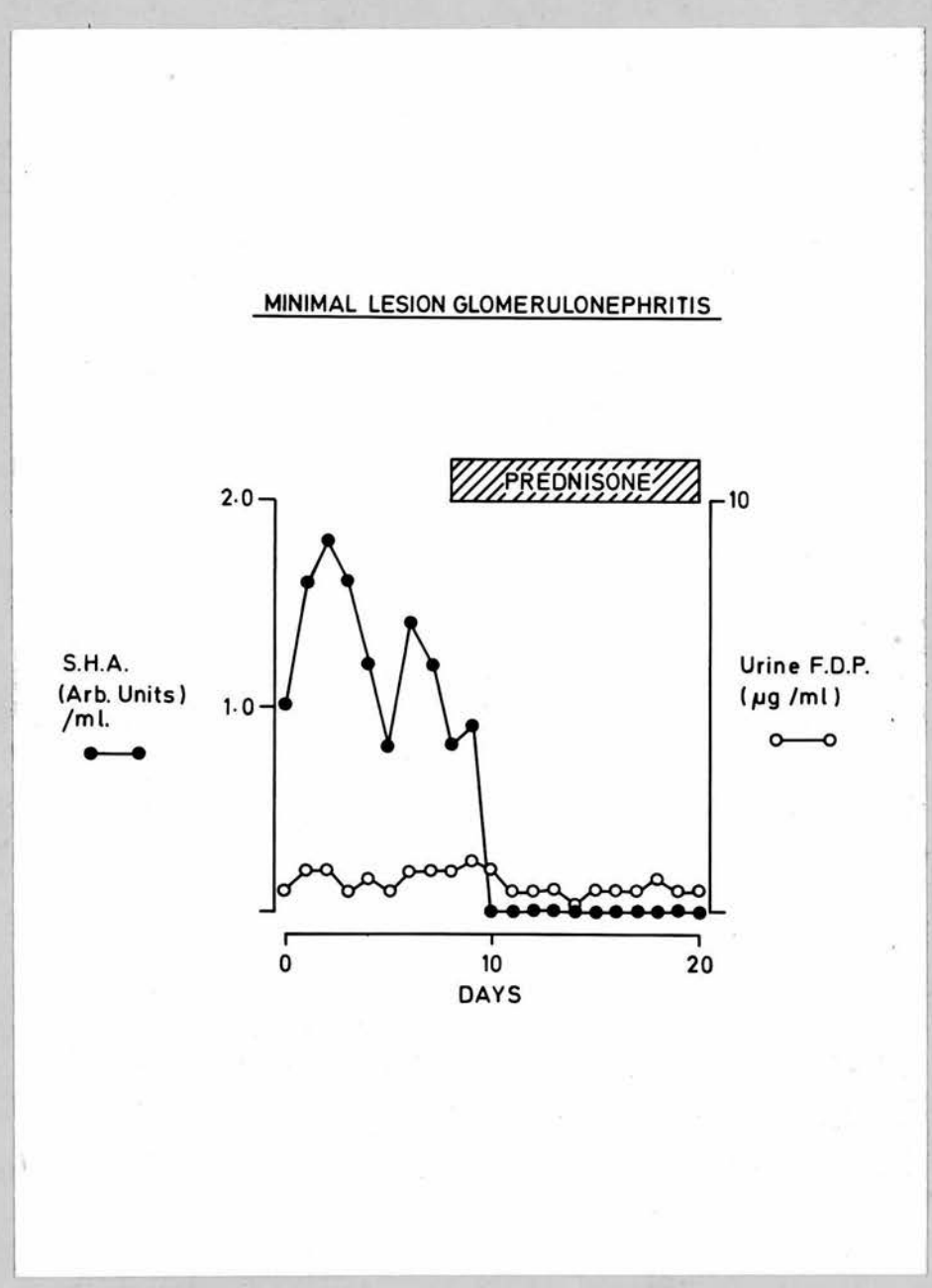

Fig. 10 The pattern of urinary F.D.P. and heterophile (sheep) haemagglutinin in a patient with minimal lesion glomerulonephritis showing less than 2 g/ml of urine F.D.P. but abundant heterophile haemagglutinin excretion. The haemagglutinin excretion returned to normal during therapy.

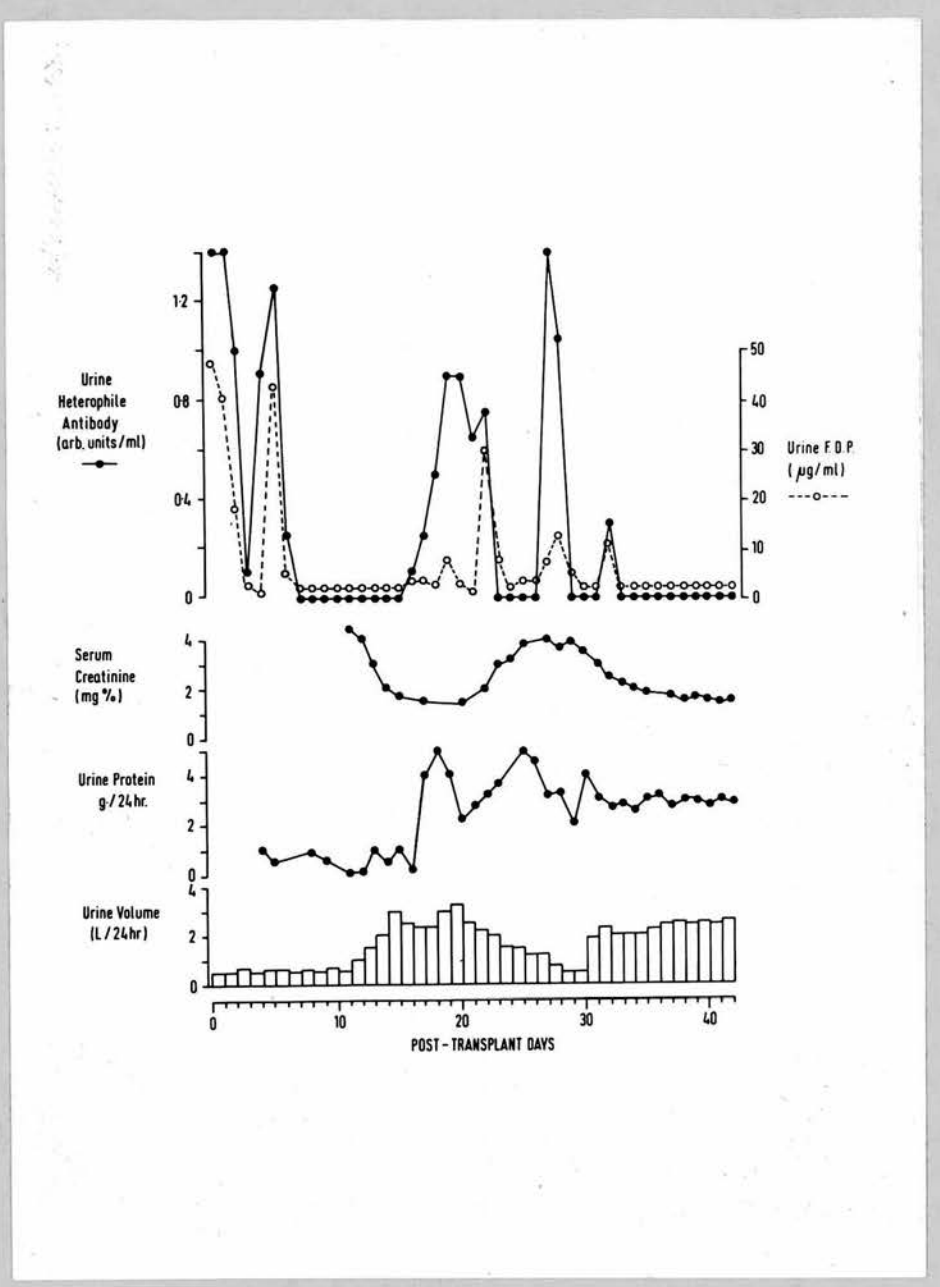

Fig. 11 Daily urine volume, urinary heterophile (sheep) antibody (haemagglutinin), F.D.P., total protein, and serum creatinine in a patient after renal transplantation followed by rejection episode, which was clinically evident on the 22nd. day, with the evidence of fall of urine volume and rise of serum creatinine. Prior to this, there was a rapid rise of both heterophile antibody and F.D.P. Fall of these parameters as the rejection episode was successfully treated.

URINARY HETEROPHILE (SHEEP) HAEMAGGLUTININ AND F.D.P. CONTENT IN PROLIFERATIVE GLOMERULONEPHRITIS DURING INDOMETHACIN ADMINISTRATION.

Although controlled trials have yet to be reported, indomethacin, an anti-inflammatory drug, has been claimed to be of benefit in the management of proliferative forms of glomerulonephritis (Michielsen et al, 1969). Clarkson et al (1972) have reported that in some patients receiving indomethacin there was a marked reduction in urine F.D.P. content during indomethacin administration. Based on previous studies (Clarkson et al, 1971) it was suggested that a reduction in disease activity might have occurred. It, therefore, seemed appropriate to study the urinary heterophile antibody content in a limited series of patients before and during indomethacin treatment.

In 5 of the 7 patients studied there was a fall in urinary heterophile haemagglutinin content during indomethacin treatment, but it did not occur as rapidly as the fall in urinary F.D.P. In all of these patients the clinical activity of the disease either improved or remained stationary during indomethacin administration. An example of such a response is shown in Fig. 12. In one of the 2 remaining patients both the urinary F.D.P. and heterophile (sheep) haemagglutinin excretion failed to be influenced by indomethacin administration (Fig. 13). This patient's renal function continued to deteriorate. Perhaps most interesting of all was the seventh patient: while there was an excellent F.D.P. response during indomethacin therapy this was not paralleled by the heterophile haemagglutinin excretion (Fig. 14). It may be significant that this patient's renal function continued to deteriorate and eventually long-term renal dialysis followed by homotransplantation was necessary.

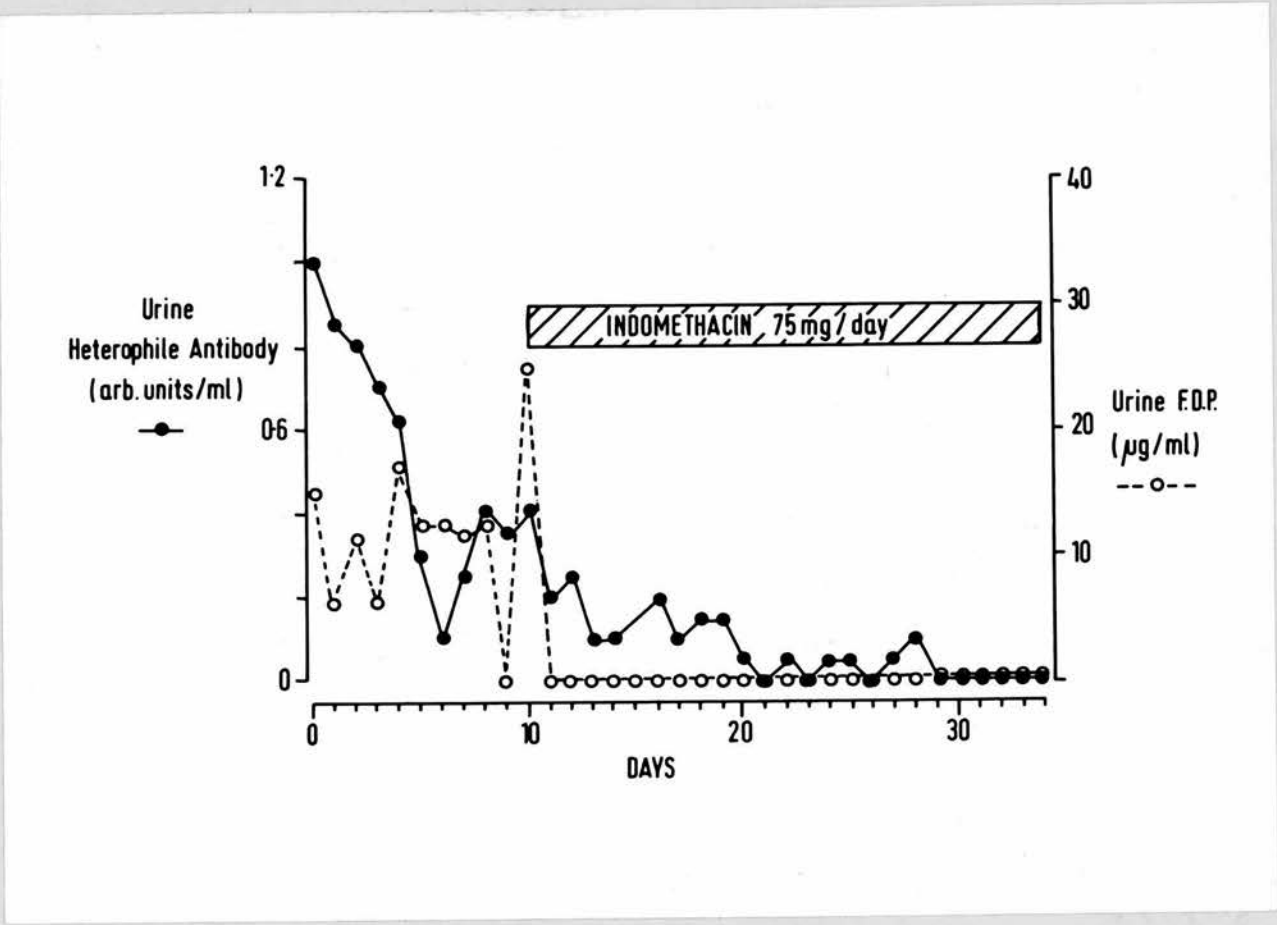

Fig. 12 The pattern of urinary heterophile (sheep) antibody and F.D.P. excretion in a patient with proliferative glomerulonephritis whose renal function improved during indomethacin administration (Note- Fall of both F.D.P. and heterophile antibody, but heterophile antibody did not fall at the same rate as the fall of urinary F.D.P.)

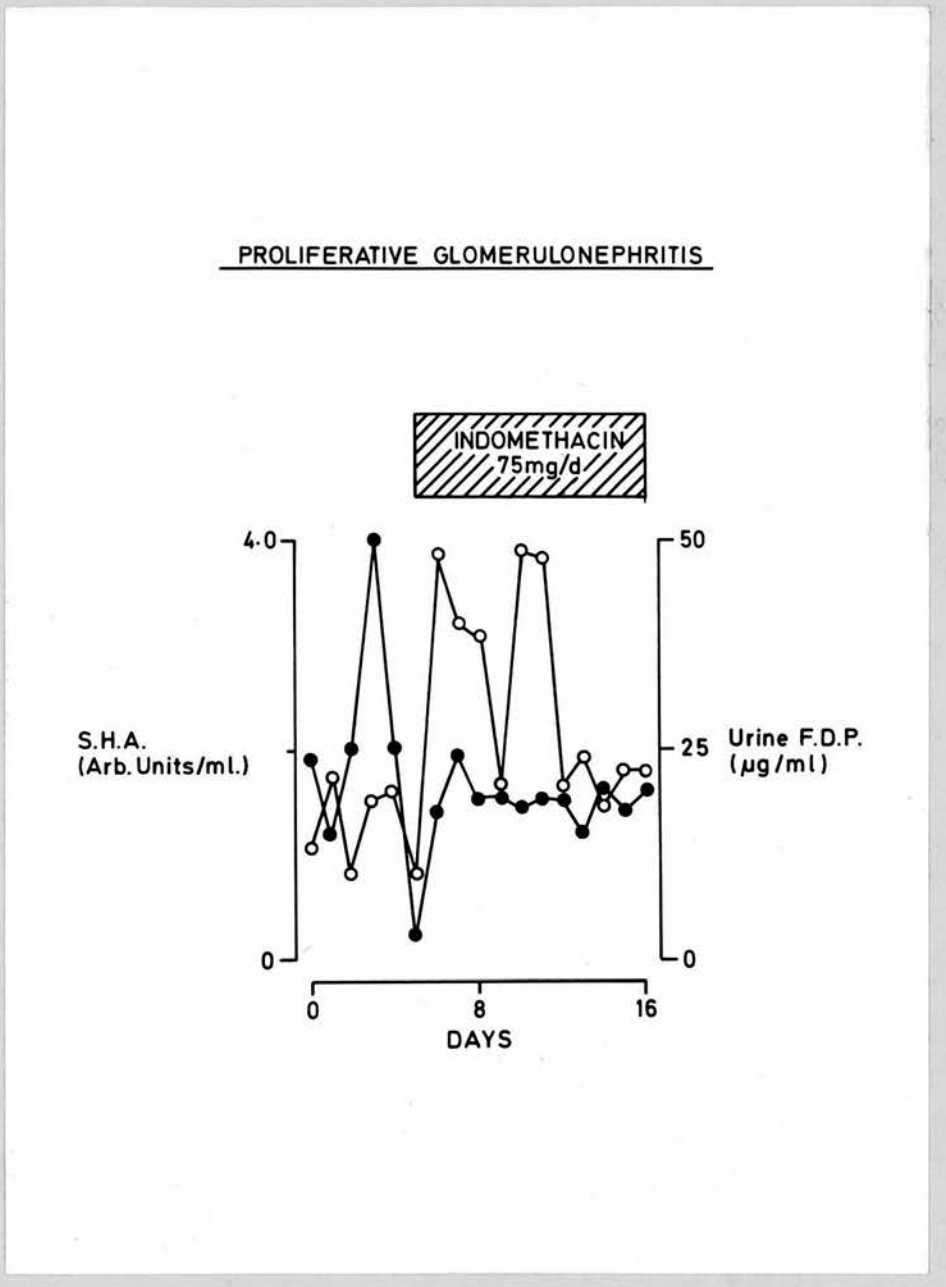

Fig. 13 The pattern of excretion of urinary heterophile (sheep) antibody and F.D.P. in a patient with proliferative glomerulonephritis during indomethacin administration whose renal function continued to deteriorate (Note - Continued excretion of both F.D.P. and heterophile antibody).

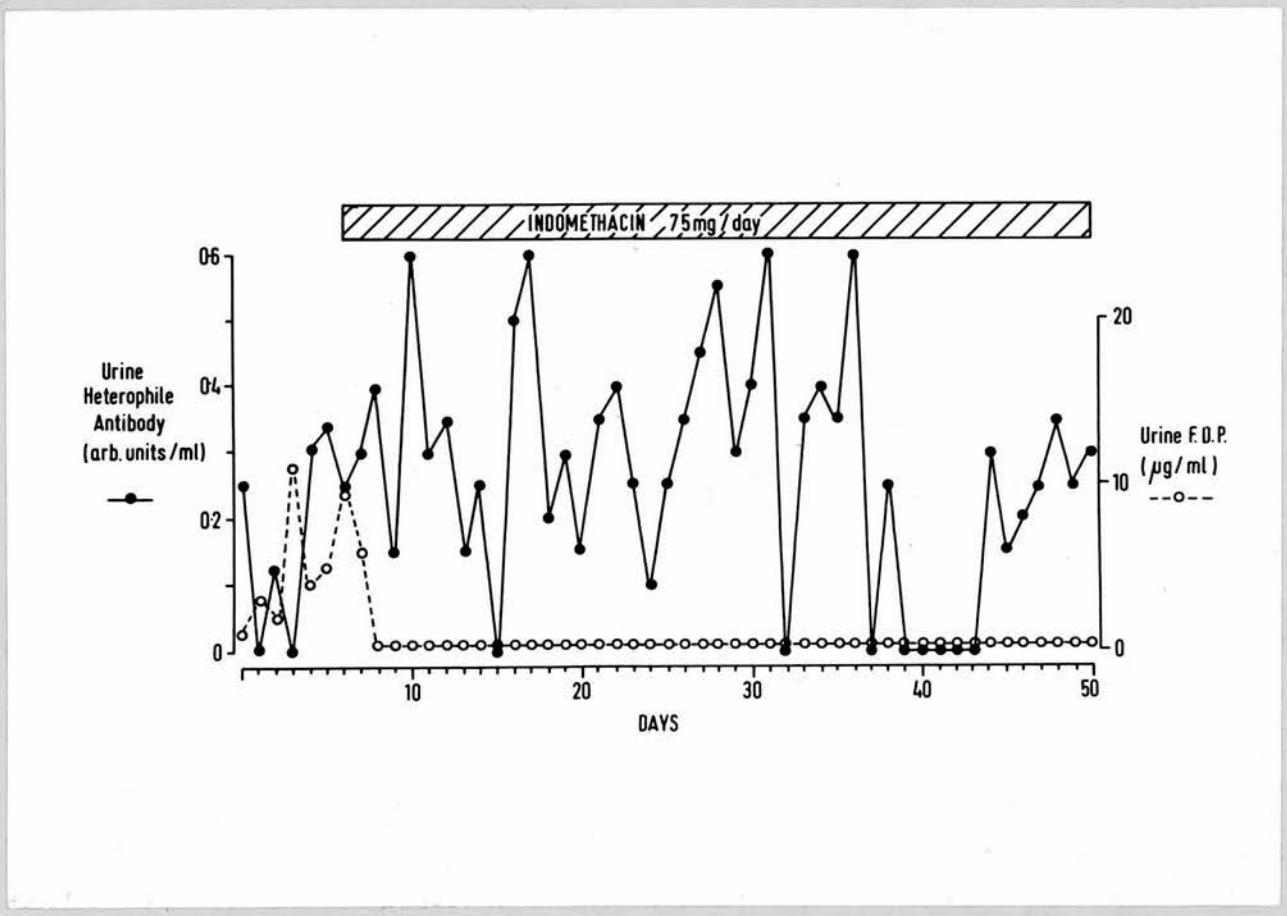

Fig. 14 The pattern of excretion of urinary heterophile (sheep) antibody and F.D.P. in a patient with proliferative glomerulonephritis whose renal function continued to deteriorate during indomethacin administration.(Note - There was an excellent F.D.P. response to treatment but continued elevation of heterophile (sheep) antibody.)

TOTAL PROTEIN, IgM, IgG AND HETEROPHILE HAEMAGGLUTININ CONTENT IN ISOLATED URINES FROM POST-TRANSPLANT AND GLOMERULONEPHRITIS PATIENTS

One of the fundamentally important aspects in understanding the presence of heterophile haemagglutinin in urine is to consider that it may arise as a simple leakage of plasma through a damaged and highly permeable glomerular basement membrane. As such, it would be of little interest and could not be regarded as being involved in an immune tissue destructive process. The first approach required to examine this possibility would be to consider the correlation between total protein content and heterophile haemagglutinin of urines.

All assays were performed on dialysed/concentrated urines (vide supra). The results of such a study are shown in Fig.15 in which it can be seen that no correlation was observed. However, a correlation was recorded between the urinary heterophile (sheep) haemagglutinin and IgG and IgM concentrations in the homotransplant (Fig 16&17) urines, and between the IgG only in the proliferative glomerulonephritis urines (Fig. 18). In minimal lesion glomerulonephritis there was also a correlation between both of these parameters (Fig. 19 & 20).

C_3 AND HETEROPHILE HAEMAGGLUTININ CONTENT IN ISOLATED URINE SAMPLES FROM POST-TRANSPLANT AND GLOMERULONEPHRITIS PATIENTS

Both active proliferative glomerulonephritis and renal homotransplant rejection are regarded as destructive processes of immunological origin and therefore likely to involve localised glomerular complement and in particular C_3 deposition (Gotoff et al, 1965; Michael et al, 1969). It therefore seemed of value to assay the urinary C_3 content in a selection of post-transplant and glomerulonephritic patients and

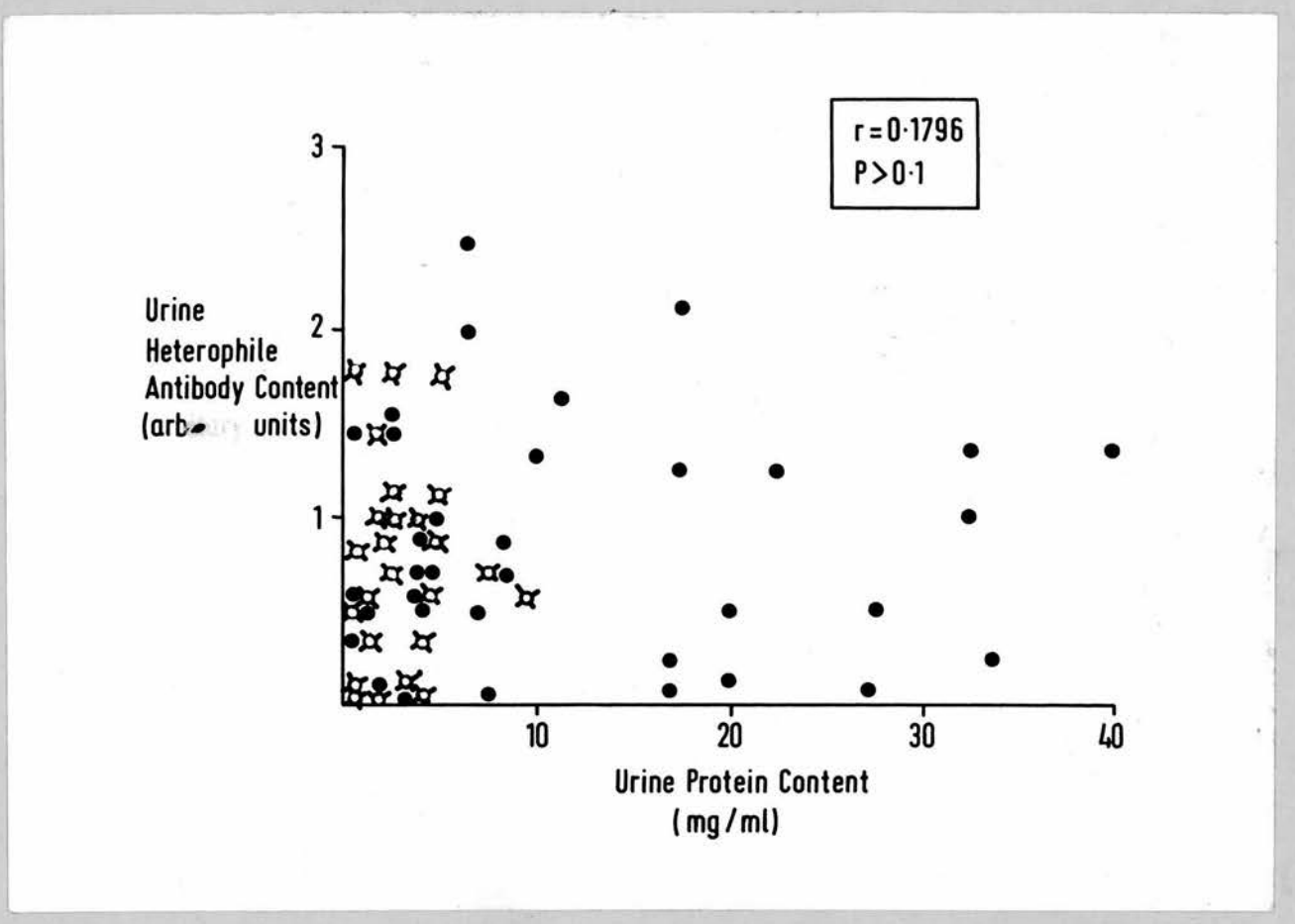

Fig. 15 Evidence of no correlation between total protein content and heterophile (sheep) antibody of concentrated urine from patients with glomerulonephrits (●) and renal transplantation (✕) [r = 0.1796, p > 0.10].

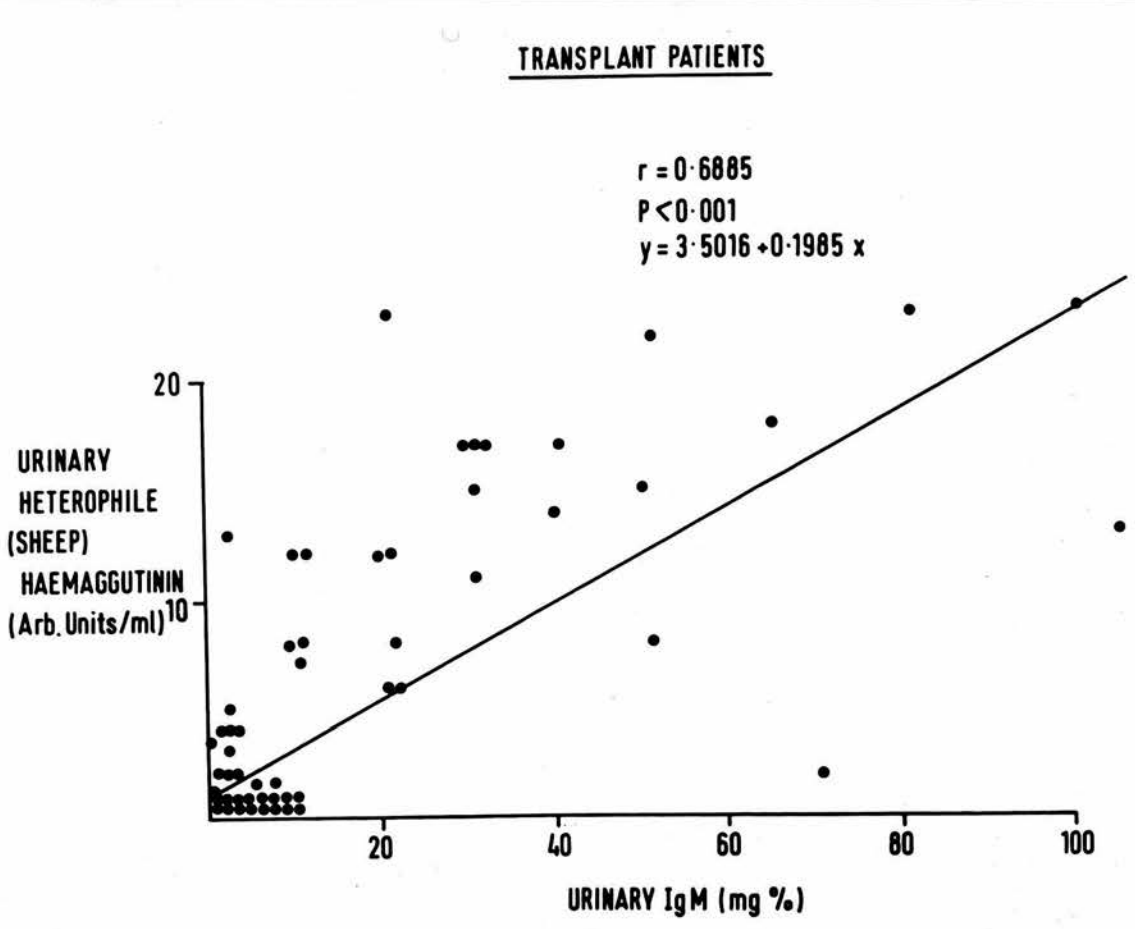

Fig. 16 A correlation between urinary heterophile (sheep) haemagglutinin and urinary IgM in transplant patients ($r = 0.6885$, $p < 0.001$).

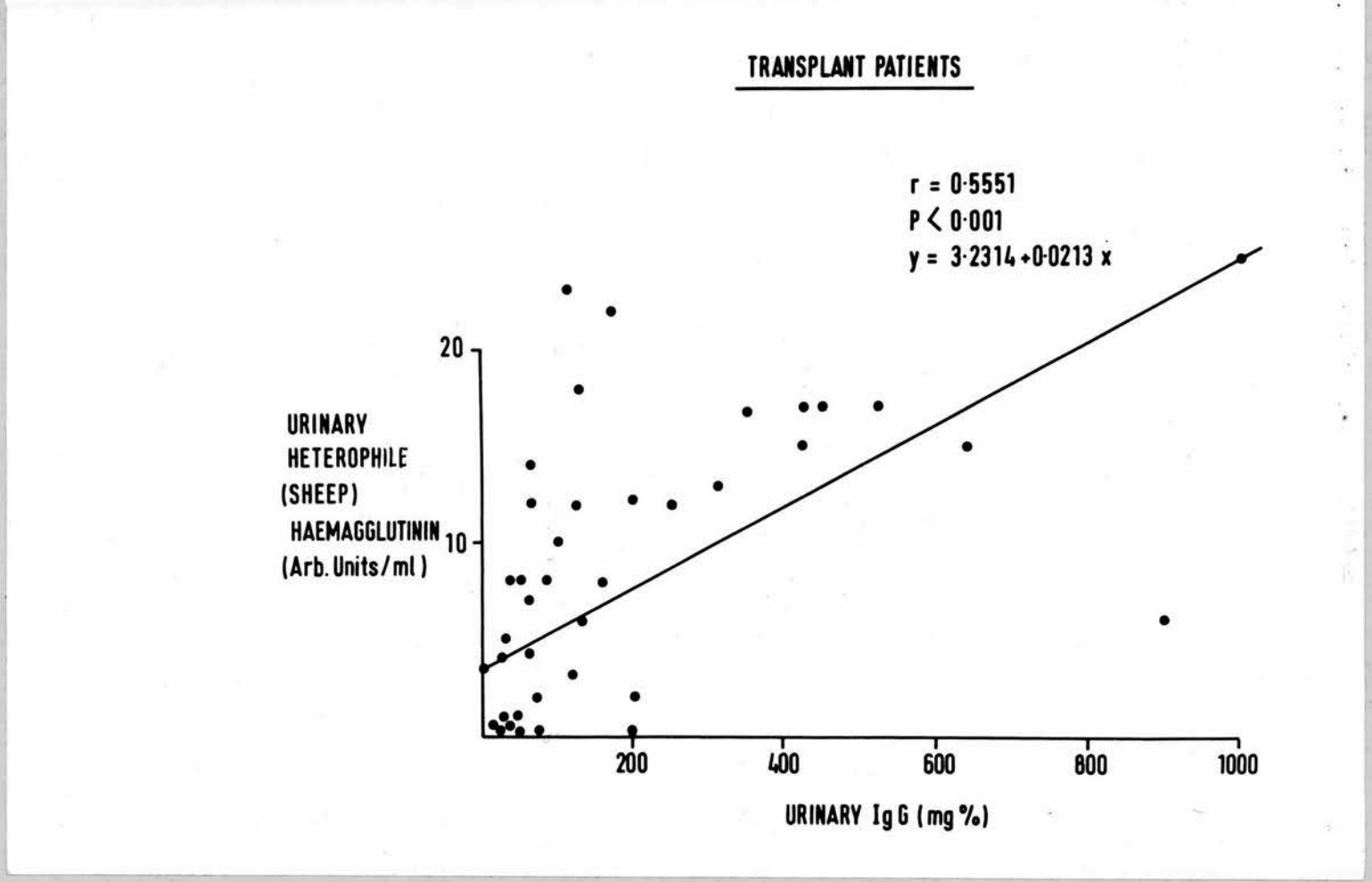

Fig. 17 A correlation between the urinary heterophile (sheep) haemagglutinin and IgG in transplant patients ($r = 0.5551$, $p < 0.001$).

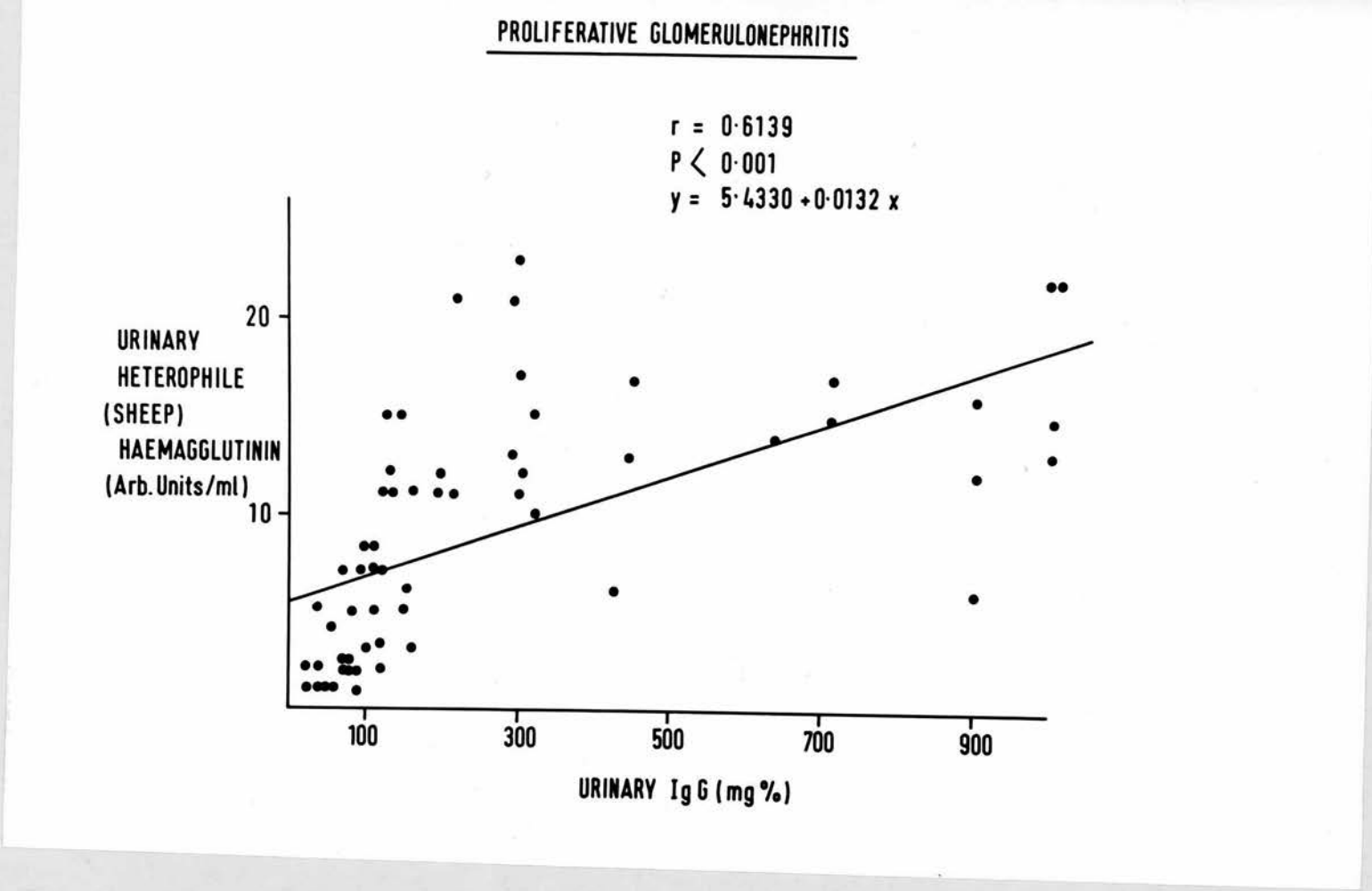

Fig. 18 A correlation between the urinary heterophile (sheep) haemagglutinin and IgG in proliferative glomerulonephritis ($r = 0.6139$, $p < 0.001$).

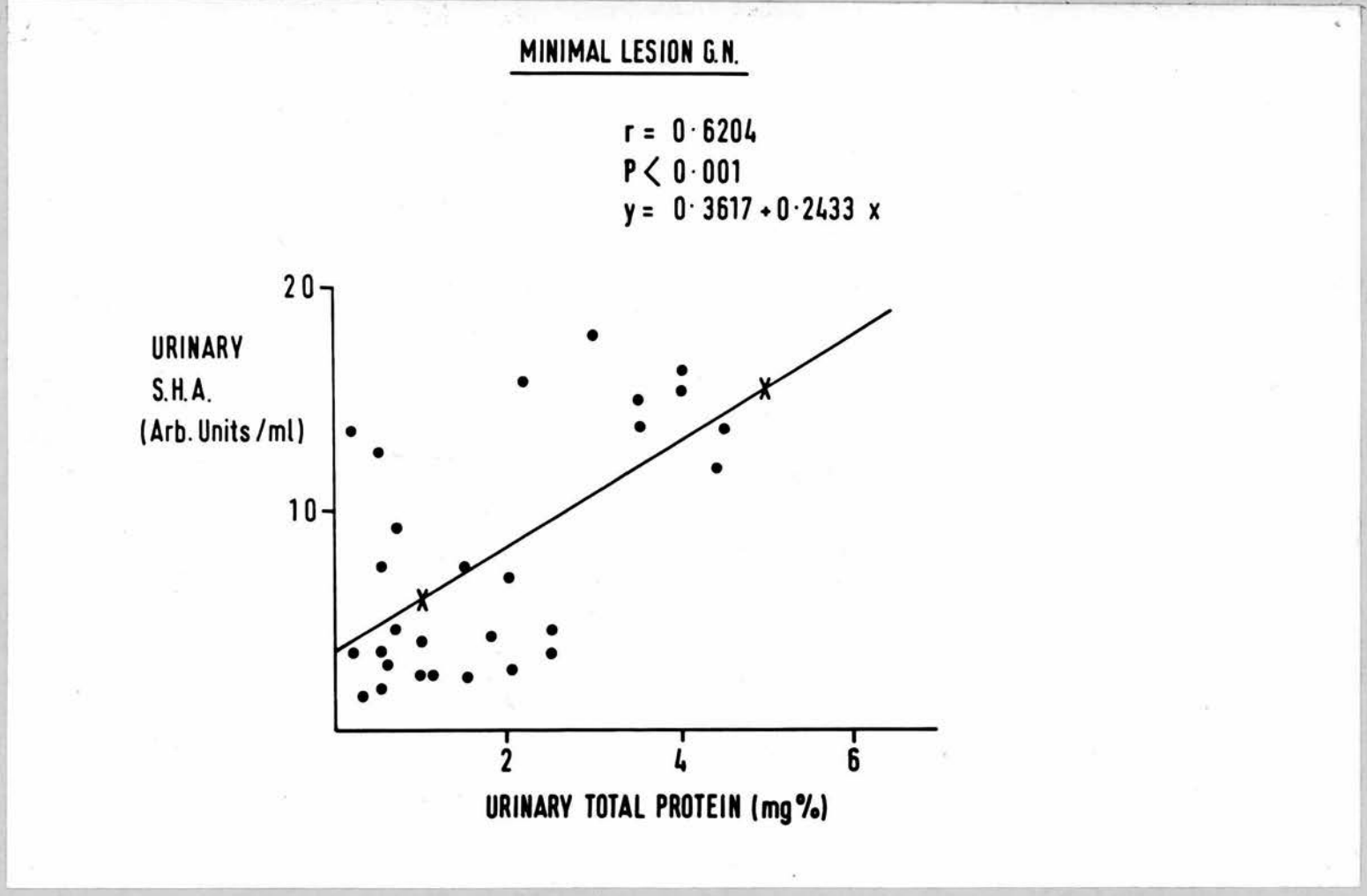

Fig. 19 A correlation between urinary total protein and heterophile (sheep) haemagglutinin in minimal lesion glomerulonephritis ($r = 0.6204$, $p < 0.001$).

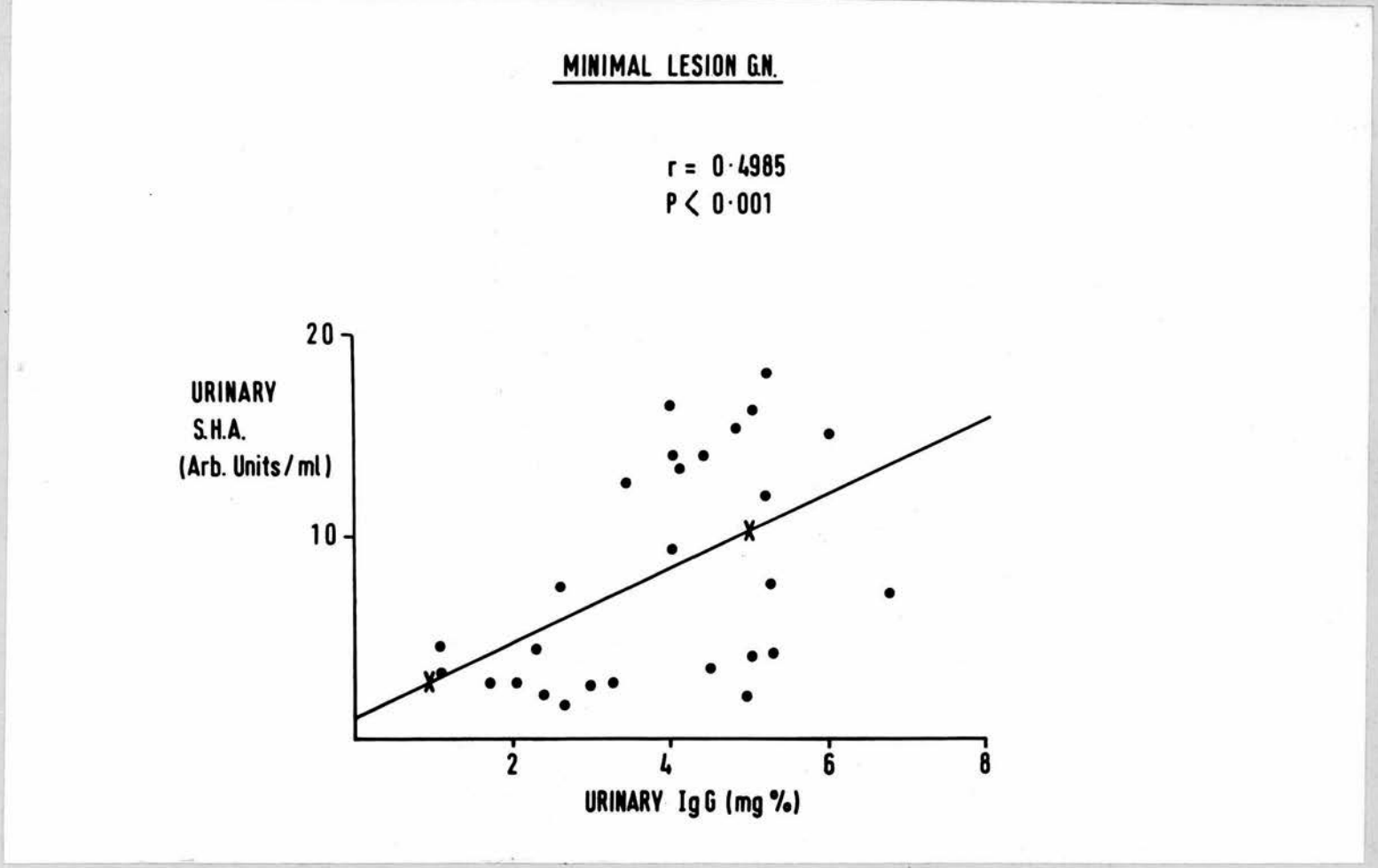

Fig. 20 A correlation between urinary IgG and heterophile (sheep) haemagglutinin in minimal lesion glomerulonephritis ($r = 0.4985$, $p < 0.001$).

correlate these findings with the heterophile haemagglutinin and F.D.P. content. It also seemed appropriate to consider the correlation between urinary C_3, IgG and IgM in these isolated samples from different patients. All assays were performed on dialysed/concentrated urines (vide supra).

The results are summarised in Table XXX. It can be seen that there was a positive correlation between the urinary heterophile (sheep) haemagglutinin, F.D.P. and C_3 content. There was also a positive correlation between the C_3 and IgM and C3 and IgG (Figs. 21 & 22).

COMBINED SERIAL STUDIES ON FIVE SELECTED PATIENTS

The apparent success of the studies on a selection of urine samples from many different patients made it worthwhile considering a detailed combined investigation on a number of consecutive urine samples from 5 highly selected patients:-

1. Minimal lesion glomerulonephritis
2. An episode of renal homotransplant rejection
3. Active proliferative glomerulonephritis failing to show an F.D.P. and heterophile (sheep) haemagglutinin response to indomethacin
4. As (3.) but showing F.D.P. and heterophile (sheep) haemagglutinin responses
5. As (3.) and (4.) but showing a satisfactory F.D.P. and unsatisfactory heterophile (sheep) haemagglutinin responses.

The results of serial assays of urine F.D.P., heterophile (sheep) haemagglutinin, IgG, IgM and C_3 on these patients are shown in Fig. 23 - 27. No significant IgM or C_3 excretion was detected in the minimal lesion glomerulonephritis, although heterophile (sheep) haemagglutinin(s) were (Fig. 23)

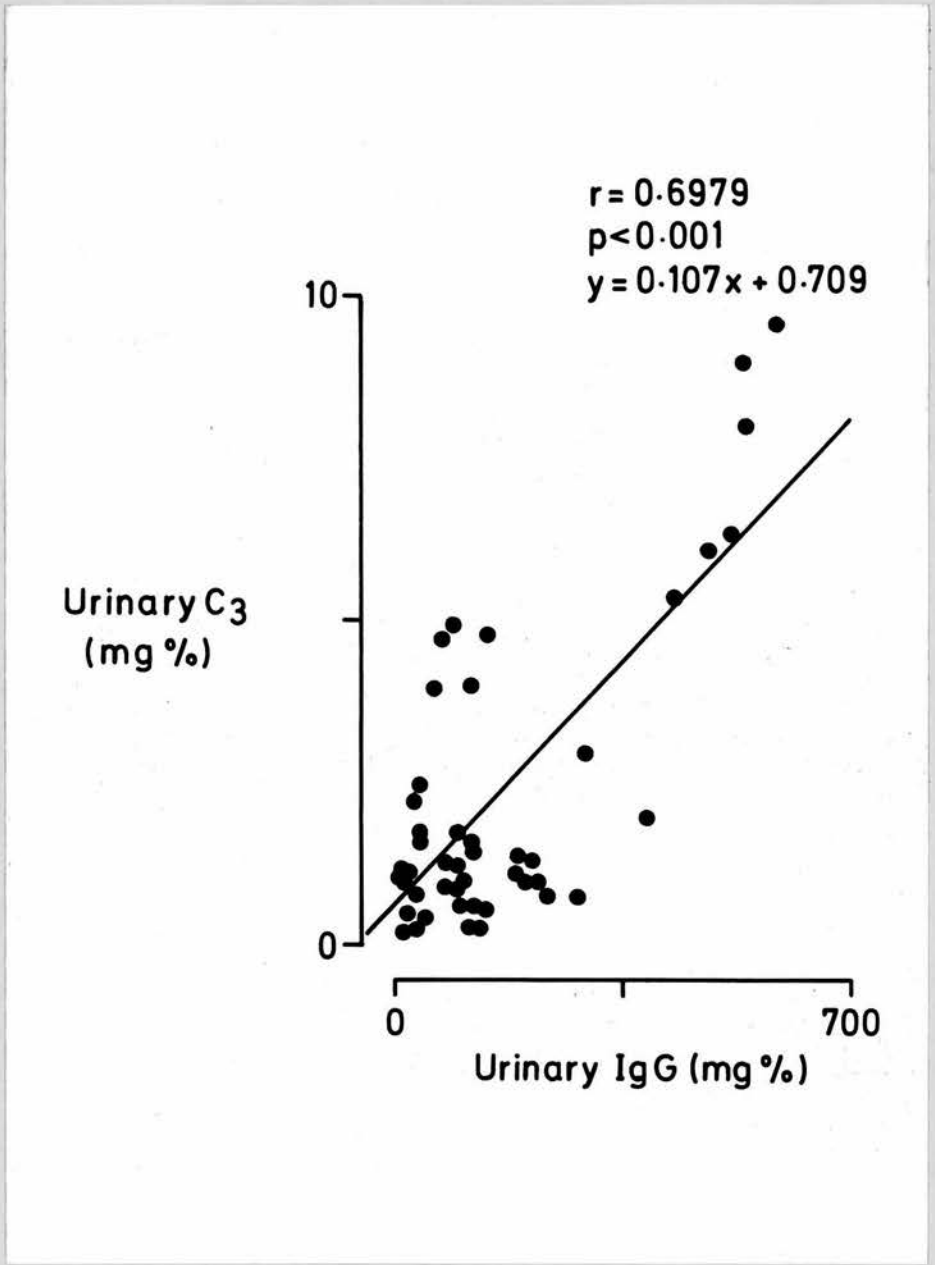

Fig. 21 A correlation between urinary C3 and IgG in isolated urine samples from post-transplant and glomerulonephritis patients ($r = 0.6979$, $p < 0.001$).

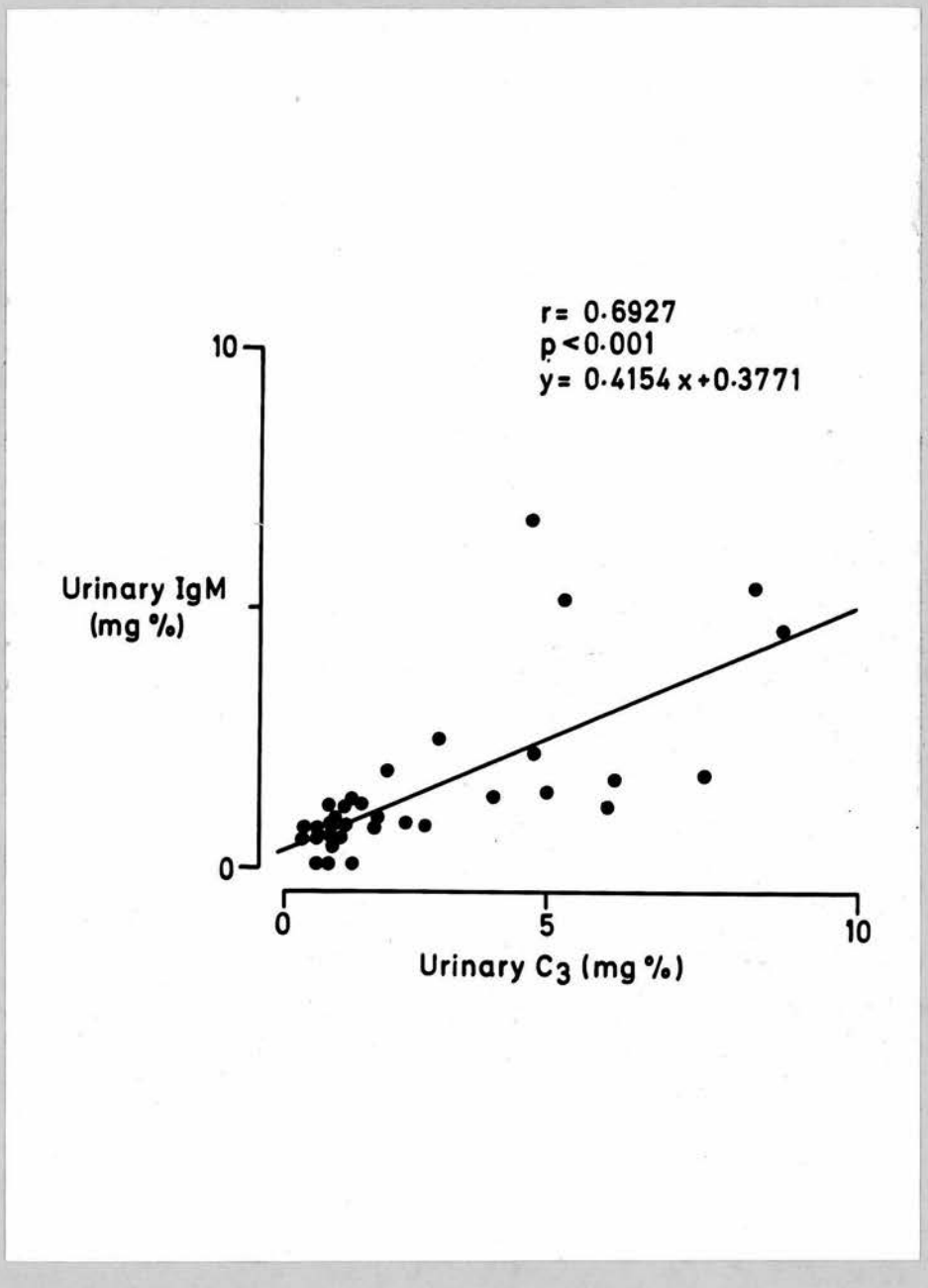

Fig. 22 A correlation between urinary C3 and IgM in isolated urine samples from post-**transplant** and glomerulonephritis patients ($r = 0.6927$, $p < 0.001$).

PARAMETERS	NUMBER STUDIED	r-VALUE	p-VALUE
C_3 / S.H.A.	96	0.8319	< 0.001
C_3 / F.D.P.	96	0.6431	< 0.001
C_3 / IgM	64	0.7291	< 0.001

TABLE XXX

Correlation between urinary F.D.P., Heterophile (sheep) haemagglutinin (S.H.A.), IgM and C_3 in a selection of isolated samples from patients with proliferative glomerulonephritis and renal transplantation.

present. The unremarkable F.D.P. content confirmed previous observations. The transplant rejection episode was associated with a elevation in all parameters assayed. (Fig. 24)

The 3 highly selected proliferative glomerulonephritis patients receiving indomethacin showed particularly interesting results. Three parameters gave parallel information, namely the excretion of heterophile (sheep) haemagglutinin, IgM and C3. Thus in the patient who showed evidence of an excellent F.D.P. response to indomethacin there was a continued excretion of heterophile (sheep) haemagglutinin, IgM and C3 (Fig. 25). This patient's renal function continued to deteriorate, as did the patient whose combined measurements showed no change during indomethacin administration (Fig. 26). The patient whose renal function improved on indomethacin treatment showed a dramatic fall in the excretion of F.D.P., heterophile (sheep) haemagglutinin, IgM and C3 (Fig. 27).

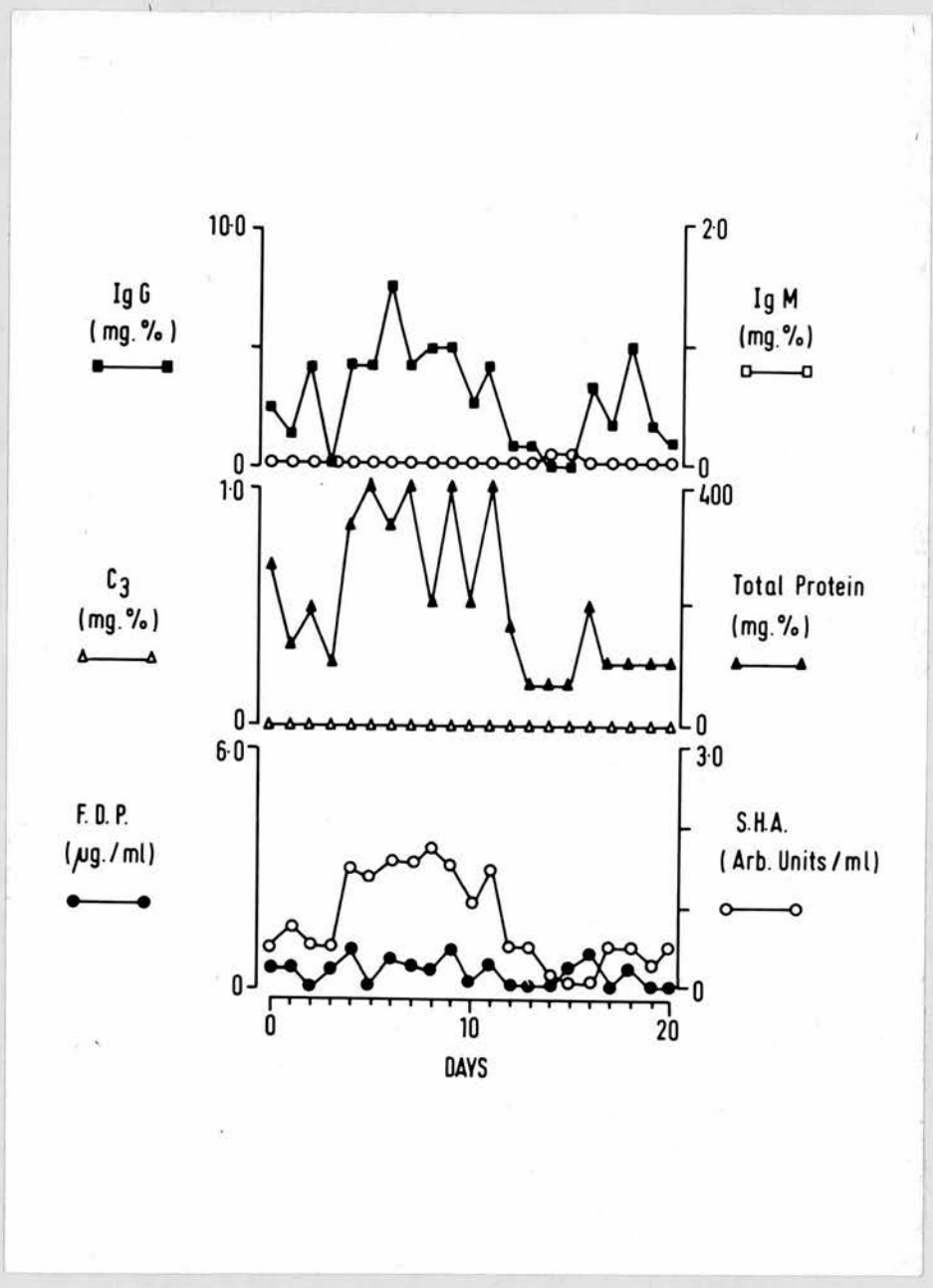

Fig. 23 Combined serial studies on urines from a patient with minimal lesion glomerulonephritis showing no detectable IgM or C3 although heterophile (sheep) haemagglutinins as well as total protein and IgG were present.

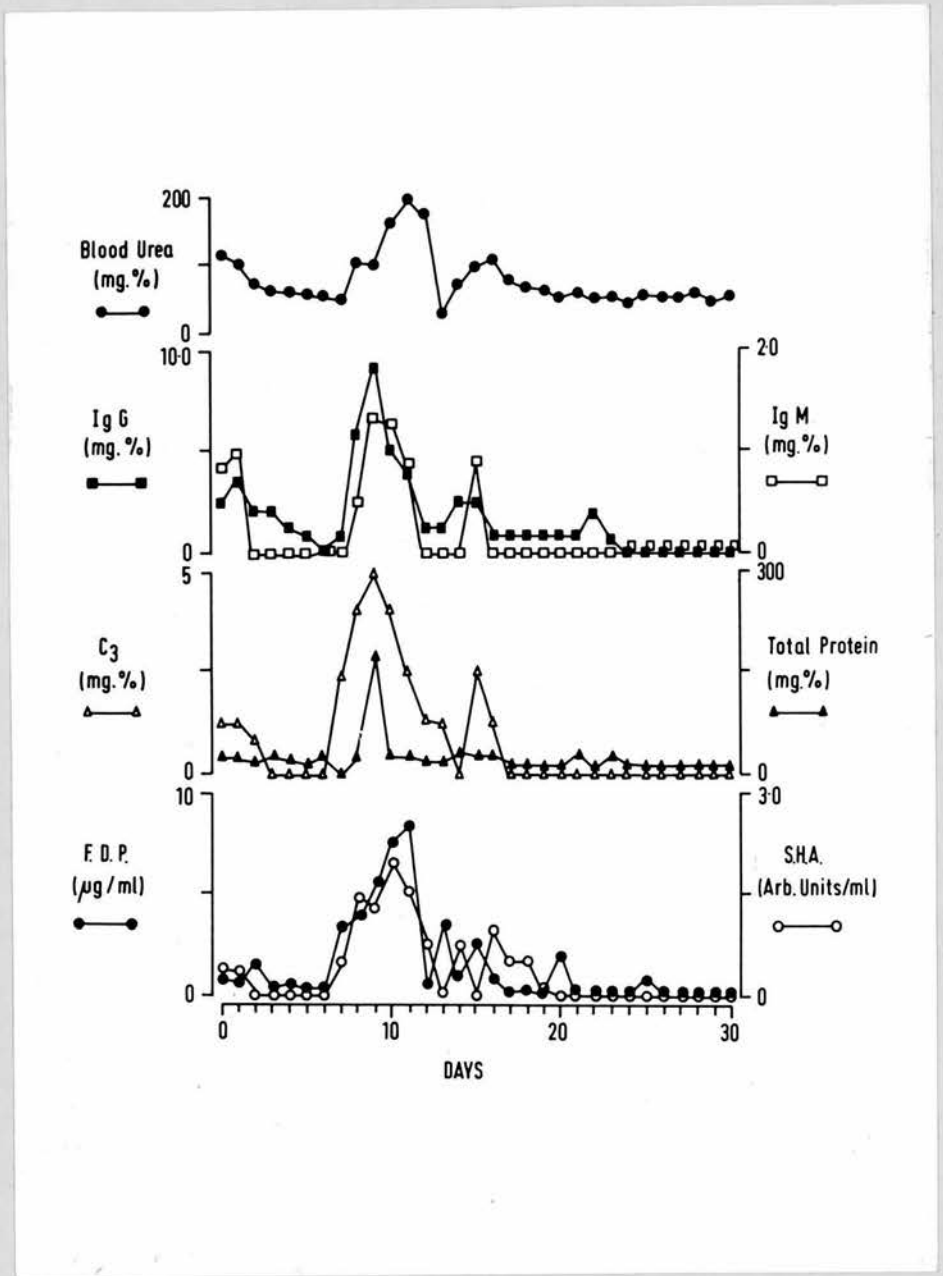

Fig. 24 Combined serial studies on urines from a transplant patient with a rejection episode. (Note - There was elevation in all parameters assayed, i.e. Heterophile (sheep) haemagglutinin, F.D.P., total protein, C3, IgM, IgG and blood urea. A fall in all parameters occurred after successful treatment).

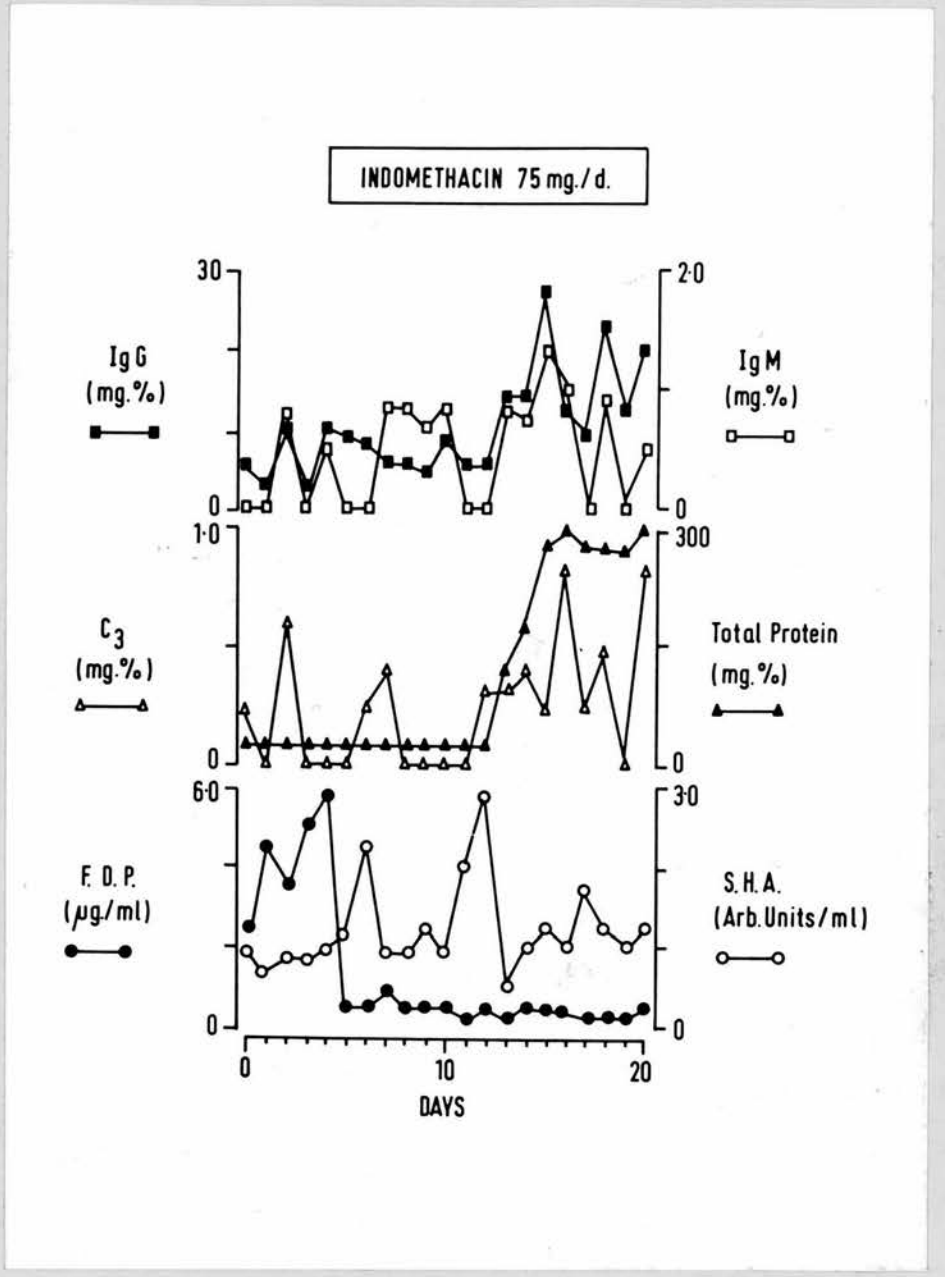

Fig. 25 Combined serial studies on urines from a patient with proliferative glomerulonephritis receiving indeomethacin showing an excellent F.D.P. response to treatment. (Note - Continued excretion of heterophile (sheep) haemagglutinin, IgM, C3, IgG and total protein. This patient's renal function continued to deteriorate.)

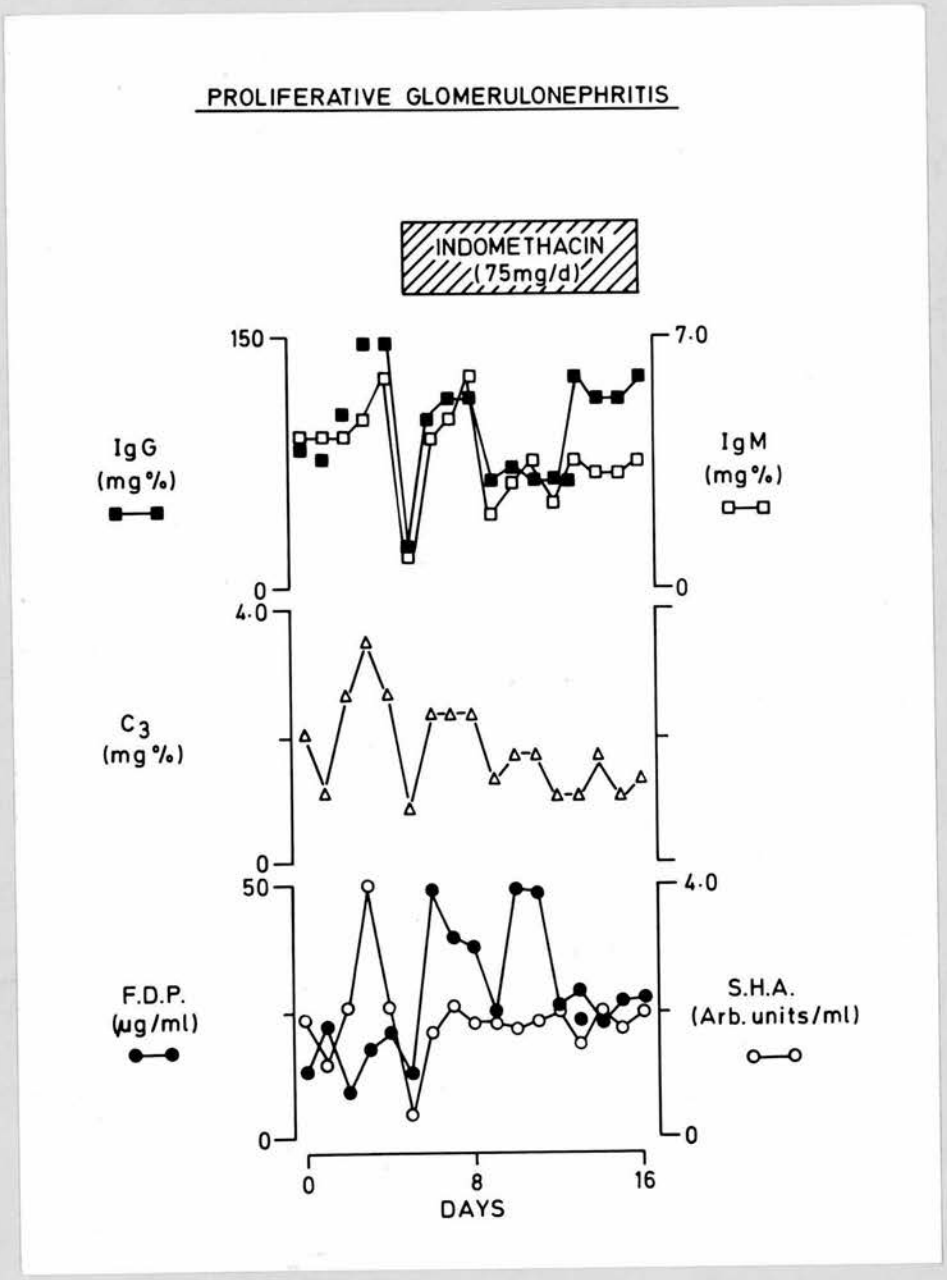

Fig. 26 Combined serial studies on urines from a patient with proliferative glomerulonephritis showing no response to treatment. (Note - Continued excretion of F.D.P., heterophile (sheep) haemagglutinin, IgM, IgG and C3).

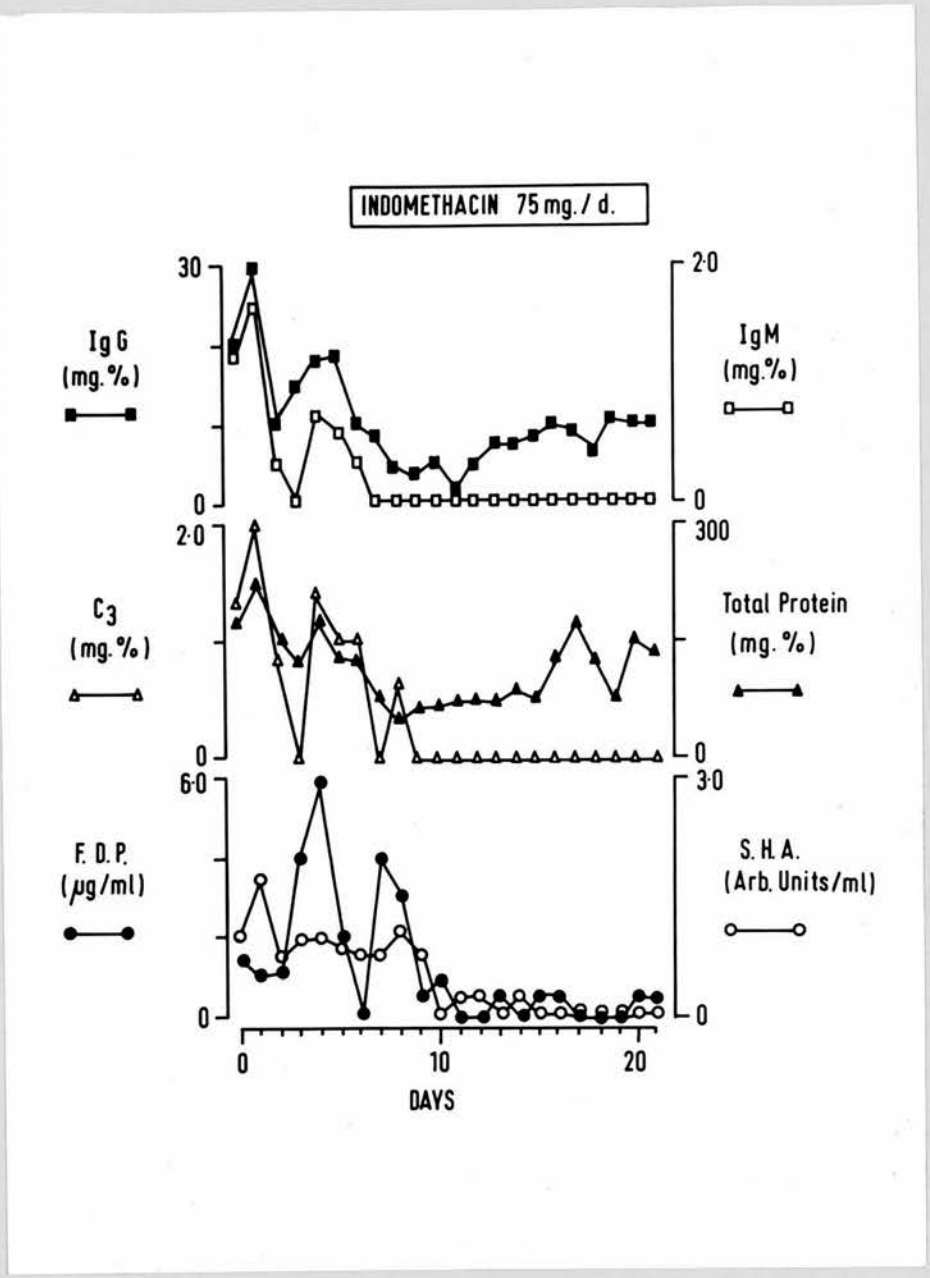

Fig. 27 Combined serial studies on urines from a patient with proliferative glomerulonephritis whose renal function improved on indomethacin treatment. (Note - dramatic fall of urinary F.D.P., heterophile (sheep) haemagglutinin, IgM and C3.)

QUALITATIVE STUDIES ON URINARY HETEROPHILE HAEMAGGLUTININ

Urinary haemagglutinins to sheep, rabbit and rat cells

Gluataradehyde fixed sheep (high reacting: Finnish Landrace, and low reacting Merino), rabbit (New Zealand White) and rat (Sprague-Dawley) erythrocytes were prepared and the titres of urinary agglutinins ascertained against a panel dialysed/concentrated urine samples, obtained from patients with glomerulonephritis (45 proliferative, 53 minimal lesion) and post-renal transplantation (30).

The results are summarised in Tables XXXI . It was observed that rabbit erythrocytes gave an overal higher titre than any of the other cells in urines from transplant and proliferative glomerulonephritis ($p < 0.001$). Moreover, rabbit cells detected agglutinins in a higher proportion of these urines tested ($p < 0.001$). Sheep (high reacting) cells appeared to react more frequently with proliferative glomerulonephritis urines, compared to rat, but the situation was reversed in the transplant urines ($p < 0.01$). In minimal lesion, however, the high reacting sheep cells detected a greater number of positive urines than either rabbit or rat cells, and the titres were accordingly higher ($p < 0.001$). Low reacting sheep cells were found to be of little value.

Urinary heterophile haemagglutinin absorption studies

Two types of absorption studies were undertaken: cross absorption using heterologous and homologous red cells (sheep, rabbit, rat and human group O, A and B) and the combined used of guinea-pig-kidney

Urinary haemagglutinins against sheep, rabbit and rat erythrocytes in glomerulonephritis and transplant

A. Proliferative glomerulonephritis

CELLS	SCORE (ARBITRARY UNITS/ML						TOTAL NUMBERS
	0	0-0.5	0.51-1.0	1.1-2.0	2.1-3.0	3.10-4.0	
Sheep high reacting	5	19	16	3	2		45
Sheep low reacting	36	7	2	0	0		45
Rabbit	0	10	19	14	2		45
Rat	15	17	11	2	0		45
B. Transplant							
Sheep high reacting	14	10	2	4	0		30
Sheep low reacting	23	5	2	0	0		30
Rabbit	0	17	6	5	2		30
Rat	12	12	1	5	0		30

TABLE XXXI

(CONTD.)

C Minimal lesion glomerulonephritis

Sheep high reacting	0	28	13	12			53
Rabbit	40	11	0	2			53
Rat	45	7	1				53

TABLE XXXI (Contd.)

emulsions and partially boiled ox red cells. This latter approach was to determine whether the reaction was a Paul-Bunnel type and the methods used were as described by Barrett (1941). A known positive Paul-Bunnel serum was used as a control.

Three urines were selected with known high titre heterophile haemagglutinins (2 proliferative glomerulonephritis and 1 post-transplant) and absorped with aliquots of all cells: 1 part urine, 1 part packed cells incubated at $4^{o}C$ overnight. After the absorption procedure they were then tested against sheep, rabbit and rat cells. The results are summarised in Tables XXXII and XXXIII which shows that high reacting sheep cells absorbed completely the agglutinin to low reacting sheep cells, little or no effect was recorded when tested against rabbit and rat cells. Individual erythrocytes absorbed completely the agglutinin of their own species. Rabbit and rat erythrocytes partially absorbed the agglutinin to each others cells, thus showing marked cross-reactivity. Human group O, A and B Rhesus negative cells failed to influence the titre recorded against sheep, rabbit and rat cells.

Absorption studies on similar urines using boiled ox erythrocytes and guinea-pig kidney gave inconclusive results. Some behaved weakly like the Paul-Bunnel positive sera whereas others failed to absorp, or showed weak absorption with guinea-pig kidney emulsions. It was concluded that their behaviour was not of the Paul-Bunnel type (Table XXXIV).

Reactivity of Urinary Heterophile Haemagglutinin after 2M Mercaptoethanol treatment.

Five selected urines (2 transplant and 3 proliferative glomerulonephritis) were treated with 2M mercaptoethanol as follows: 0.5 ml of urine was added to 0.5ml of 0.02M mercaptoethanol in iso-

Absorption studies on urinary heterophile haemagglutinin by using homologus red cells.

Type of test samples	Group Type of red cells used for absorption	Reactivity by different species erythrocytes.		
		Sheep (H.R.)	Rabbit	Rat
P.G.N.-1	A-ve	10	12	8
	B-ve	10	13	8
	O-ve	10	12	7
	Control	12	15	10
P.G.N.-2	A-ve	12	13	10
	B-ve	12	14	10
	O-ve	12	13	10
	Control	13	14	11
TX. - 1	A-ve	12	18	11
	B-ve	11	18	12
	O-ve	12	19	11
	Control	14	20	12

H.R. - indicates high reacting.
P.G.N. - indicates Proliferative glomerulonephritis
TX. - indicates Transplant

TABLE XXXII

Absorption studies on urinary Heterophile Haemagglutinin by using heterologus red cells.

Type of test sample	Types of cells used for absorption	Reactivity by different Species of erythrocytes			
		Sheep (high reacting)	Sheep (low reacting)	Rabbit	Rat
P.G.N.-1	Sheep (H.R.)	0	0	12	8
	Sheep (L.R.)	4	0	12	8
	Rabbit	10	2	0	4
	Rat	9	2	6	0
	Control	12	2	12	8
P.G.N.-2	Sheep (H.R.)	0	0	10	7
	Sheep (L.R.)	2	0	10	7
	Rabbit	10	2	0	7
	Rat	10	2	3	0
	Control	10	2	10	7
TX.-1	Sheep (H.R.)	0	0	17	8
	Sheep (L.R.)	4	0	17	8
	Rabbit	10	2	0	5
	Rat	10	2	9	0
	Control	12	3	20	10

TABLE XXXIII

H.R. - High reacting
L.R. - Low reacting
P.G.N. - Proliferative glomerulonephritis
TX. - Transplant

Differential absorption for Paul-Bunnel antibody in the urine of Proliferative glomerulonephritis and Transplant

	Control	After absorption with guinea-pig kidney emulsions	After absorption with boiled ox red cells
P.G.N.-1	20	20	13
P.G.N.-2	15	12	2
P.G.N.-3	22	22	22
P.G.N.-4	12	10	0
P.G.N.-5	23	22	15
P.G.N.-6	12	8	8
P.G.N.-7	8	8	8
P.G.N.-8	10	8	8
P.G.N.-9	4	4	4
P.G.N.-10	4	4	2
TX.-1	13	12	6
TX.-2	8	8	4
TX.-3	12	12	4
TX.-4	4	2	0
TX.-5	2	2	0
Normal Serum	18	0	13
Control Paul-Bunnel Serum	2560	2560	1280

TABLE XXXIV

osmotic phosphate buffer (pH 7.4). A control, using the test material plus phosphate buffer alone was also incorporated. Both were incubated at 37°C for 2 hours and dialysed overnight against phosphate-saline buffer at 4°C. The samples were then tested against glutaraldehyde fixed sheep, rabbit and rat cells. The results are summarised in TableXXXVwhich suggests that the heterophile haemagglutinins in the 2 transplant urines were mainly IgM whereas the bulk in the three active proliferative glomerulonephritis urines appeared to be IgG.

Taken toghether these qualitative studies on the urinary heterophile haemagglutinin content in transplantation and active proliferative glomerulonephritis suggest that there is no single haemagglutinin involved but several. The reactivity to rat cells excludes Forssman antibody. Finally, future studies should perhaps turn to the use of rabbit erythrocytes although high reacting sheep cells may continue to be of choice in the minimal lesion type of glomerulonephritis.

Reactivity of urinary heterophile (sheep, rabbit and rat) haemagglutinins (scores) before and after 2M mercaptoethanol treatment

Urines	Sheep		Rabbit		Rat	
	Before	After	Before	After	Before	After
TX. 1	12	2	18	2	13	2
TX. 2	6	0	8	0	7	2
P.G.N. 1	8	8	8	7	4	4
P.G.N. 2	10	7	8	8	4	4
P.G.N. 3	4	0	4	0	0	0

TABLE XXXV

CHAPTER 12

DISCUSSION

It is an interesting fact that during the period when studies were reported on serum F.D.P. in renal disease, the results were controversial and disappointing. It now seems almost certain that the primary problems were the marked dilution effect after the renal venous blood mixed with the rest of the circulating blood volume, the known powerful mechanisms for clearing systemaically circulating F.D.P. and the probability that much of the intra-renal fibrin deposition is likely to be extravascularly sited. By introducing urinary estimations of F.D.P., however, the Edinburgh group, using the human cell preparations described in this Thesis, were the first to show a close correlation between disease activity in the proliferative forms of glomerulonephritis, as assessed biochemically and histologically, and the excretion of F.D.P. (Clarkson, et al, 1971; Davison et al, 1973). They were also able to demonstrate, in some patients, dramatic responses to therapy (Clarkson et al, 1972). Thus marked changes in urinary F.D.P. excretion were recorded which were not evident from serum estimations. These observations also appeared to be valid in the rejecting renal homotransplant situation (Clarkson,

et al, 1970).

In what, it is proposed is essentially a preliminary study, described in this section, the same pattern of events has emerged, although a quite different parameter has been quantitated. Thus, all previous investigations directed to the assay of heterophile haemagglutinins in the renal transplant rejection situation have been concerned with serum values. These results have also proved to be disappointing and controversial (Rapaport et al, 1968; Tiong and Morris, 1972). It is also of interest that a recent report has included the finding of increased serum heterophile haemagglutinin titres in occasional patients with 'nephritis' (Svehag et al, 1973). Once again, it would seem that the application of urinary heterophile haemagglutinin assays, rather than serum, has revealed considerably more information than hitherto available. Moreover, the approach of correlating this data with urinary F.D.P. excretion has also been of interest.

The initial results of this study would suggest that in proliferative glomerulonephritis and following human renal homotransplantation there is an overal parallelism between the excretion of urinary F.D.P. and heterophile haemagglutinins. However, this does not appear to apply in all situations and the one which has clearly been demonstrated in this study is the response to the administration of indomethacin in proliferative

glomerulonephritis. It has been shown, conclusively, that there is a subgroup of patients whose F.D.P. response appears to be satisfactory, and as such would, according to previous studies (Clarkson et al, 1972) be expected to stabilise or improve their renal function. In the event this did not occur and it was noted that their abnormal heterophile haemagglutinin excretion continued, unaffected by the indomethacin.

From a clinical point of view this latter observation may prove to be of some importance, as it may represent a new subgroup of patients whose therapeutic requirements may be significantly different from other patients. Thus, with respect to the total data now available from Edinburgh on the responses to indomethacin in proliferative glomerulonephritis at least 3 major subgroups have emerged :-

Group 1 - Failure of F.D.P. and heterophile haemagglutinin excretion to fall.

Group II - Parallel fall in both parameters.

Group III - Fall in F.D.P. but continued excretion of heterophile haemagglutinin.

If these subgroups are confirmed and if the clinical impression that Groups 1 and 111 are destined to continued renal functional deterioration, then future trials on indomethacin in proliferative glomerulonephritis should be so designed to exclude them. The existing technology makes this possible.

The 'Group III phenomenon' is also of considerable theoretical interest, for it would suggest that intra renal fibrin deposition may not be the key aetiological factor in the progressive glomerular damage in man, and that the animal studies of Vassalli and McCluskey (1964) and Humair et al, (1969a, 1969b) may not be a satisfactory model. This conclusion, it is suggested, is supported by the collateral observation of continued C3 and IgM excretion in this type of patient during indomethacin administration.

There are other significant practical conclusions which may be drawn from these studies, that are of particular relevance to those responsible for providing laboratory services to Nephrology Units. From the laboratory point of view there are certain advantages to the serial monitoring of patients' heterophile haemagglutinin excretion rather than F.D.P. The former technique is a direct one-stage agglutination reaction and as such is much easier to perform and control than the complicated and laborious two-stage haemagglutination inhibition immunoassay required for F.D.P. determinations. Moreover, the reagents required for urinary heterophile haemagglutinin estimations are readily available in any laboratory area, whereas good quality reagents for the T.R.C.H.I.I. (F.D.P.) are so difficult to prepare that many laboratories have abandoned this approach and sought refuge in obtaining commercial supplies. Such a move has proved

to be costly, and excessively so when it is appreciated that meaningful data on individual patients can only be obtained from serial studies on a large number of urine samples. Finally, preliminary studies in this laboratory have shown that unlike the T.R.C.H.I.I. (F.D.P.) the heterophile haemagglutinin technique can be readily modified to suit automation using a Technicon Single Channel Autoanalyser. It is believed that the further development of this approach will enable an individual laboratory to assay urinary heterophile haemagglutinins at a rate of 30 samples per hour. Thus, although it must be emphasised that in the foreseeable future it will still be necessary to assay urinary F.D.P., as the data presented in this Thesis are essentially preliminary in nature, there are already sufficient reasons why those responsible for laboratory work might wish to see a conversion from urinary F.D.P. to heterophile haemagglutinins. Moreover, from a clinical standpoint the latter may prove to be a more relevant marker of on-going immune damage.

When considering the origins of urinary heterophile haemagglutinin in proliferative glomerulonephritis and during transplant rejection the author finds himself on less certain ground. Certainly the data presented demonstrates that the haemagglutination does not arise from the cross-reaction of urine F.D.P. Moreover, studies on purified preparations of F.D.P. (fragments D and E, kindly supplied by Doctor P. Gaffney) did not

agglutinate sheep, rabbit or rat cells. The absence of a correlation between the urinary total protein content and heterophile haemagglutinin titre, and the good correlation between the urinary IgG and IgM and heterophile haemagglutinin titre, combined with the knowledge that in sera this reaction is related to the IgG and IgM fractions would support the conclusion that the urinary heterophile haemagglutinin is indeed a heterophile antibody. But, is its appearance related to a leaking basement membrane, and as such is a feature of 'dilute plasma', or does it arise following the known glomerular deposition of IgG and IgM in these conditions (Berger, 1969; McCluskey, 1971; Macanovic et al, 1972). To conclude that the latter is at least part of the mechanism in proliferative glomerulonephritis and renal transplant rejection implies that urine heterophile antibody does not arise entirely as a passive leak constituent in the proteinuria, but is closely related to the immune damage process.

Perhaps the most useful studies, in this project, which give at least a clue to this problem are those in which serial estimations of urinary F.D.P., heterophile antibody, IgG, IgM and C3 were performed on a highly selected group of patients. Thus it is proposed to discuss this data in some detail.

The complement system consists of 9 main proteins and a number of cofactors which when activated, through cell bound components, leads to cytolysis (Humphrey and Dourmashkin, 1965) and/or the release of soluble factors with biological activity. Thus activation leads to all the features of inflammation. 'Classical' activation occurs following antigen-antibody combination, but an 'alternate pathway' exists (Gotze and Muller-Eberhard, 1971) and recent studies would

suggest that this pathway may indeed be important in certain types of proliferative glomerulonephritis, (Hunsicker et al, 1972). The evidence that complement is involved in human glomerulonephritis is good (Lancet, 1972). This includes immunofluorescent techniques which have shown it to be present in the glomerulus during active disease, and in particular the third component of complement, known as C3. Thus at the present time, histological data suggests that both in active proliferative forms of glomerulonephritis and in the rejecting renal allograft, IgG, IgM, C3 and fibrin are deposited in the renal glomerulus, (McKenzie and Whittingham, 1968; Porter et al, 1968; Berger, 1969; McCluskey, 1971 and Macanonvic et al, 1972).

It is now well established that urinary proteins are composed of antigens of different size and origin: some related to serum proteins, others kidney or renal tract proteins (Manuel and Revillard, 1970). Moreover, experiments have shown a glomerualr sieve effect and tubular reabsorption influences the final composition of urinary proteins. In general, proteins with a molecular weight of less than 88,000 pass, without major alterations, through the glomerular membrane , whereas macromolecules are excluded and not normally found in urine. Thus albumin, IgA (secretory) and intact IgG represent the major part of plasma proteins detected in normal urine (Poortmans and Jeanloz, 1968), whereas IgM has not been found (Turner et al, 1970), and some of the IgG has undergone degradation to Fc type of fragments (Berggard and Bennich, 1967).

The results of the serial studies on the patients with proliferative glomerulonephritis receiving indomethacin would suggest that the presence of heterophile antibody, IgM and C_3 are largely related

to an active disease process rather than passive leakage of plasma proteins. This is deduced by their rapid fall in urinary excretion during indomethanin administration in the presence of an altogether less dramatic fall in IgG and total protein excretion. It therefore seems unlikely that the dramatic fall in heterophile antibody, IgM and C3 was due to a sudden change in protein selectivity. This is supported by the finding of continued unchange excretion of α_2-macroglobulin, which has a molecular weight close to IgM. Moreover, the molecular weight of IgG is close to that of C3 and if the changes arose from selectivity alterations alone then the fall in C3 should have been paralleled by IgG; this did not occur. Thus it is proposed that the urinary excretion of heterophile antibody in proliferative glomerulonephritis and in the rejecting renal allograft is likely to be part of an immune intrarenal destructive process, and, moreover, as such may be a more reliable index of this activity than urinary F.D.P. excretion.

One of the many interesting further questions which arises from these studies is whether the heterophile antibodies found in the urine are directed against partially damaged renal tissue, and as such give rise to further and progressive renal tissue destruction. Such a concept is not new, as the role of a secondary autonomous phase with the production of autoantibodies against autologous damaged kidney antigens has been already postulated (Houba et al, 1971), although without evidence. This hypothesis for heterophile antibody gains further acceptance in the light of recent studies which show that serum heterophile (rat) antibody in patients following renal transplantion can be absorped by human kidney tissue (Svehag et

al, 1973). Moreover, Forssman antibody, when inoculated into normal guinea pigs produces vascular inflammatory lesions. (Taichman and Chi-Cheng Tsai, 1972). This tissue damaging process appears to be complement mediated (May and Frank, 1972). Thus it seems possible that the heterophile antibodies may be directed against altered glomerular antigens and that in turn the interaction of these antigens with the heterophile antibodies may be part of a vicious circle of destruction.

One of the further interests in this study was the position of the heterophile antibody in the minimal lesion glomerulonephritis. It is assumed that this arises from leaking IgG and is not related to a specific immune process.

The qualitative studies on the urinary heterophile haemagglutinin would suggest that they are IgG and IgM. The results of using sheep, rat and rabbit erythrocytes strongly imply a heterogenicity of antibodies with respect to antigenic determinents. The absorbtion studies, however, were not clear cut, and so do not confirm previous results on serum heterophile antibodies in renal transplantation when it was shown that there was a significant cross-reaction with the B antigen on human red cells (Svehag et al, 1973).

S E C T I O N IV

FINAL CONCLUSIONS

CHAPTER 13

FINAL CONCLUSIONS.

There have been three main themes in this project: the development of a new T.R.C.H.I.I. (F.D.P.) in which human red cells replace sheep erythrocytes; the development of a standardised assay for urinary heterophile haemagglutinins; and the application of these two technologies in glomerulonephritis, and renal transplantation.

In the first series of studies it was discovered that by the introduction of glutaraldehyde, instead of formaldehyde, satisfactory human red cells could be prepared for the T.R.C.H.I.I. (F.D.P.). This development provided a key reagent for extensive studies on urinary F.D.P. In the second techological study techniques were developed which provided stable and reproducible reagents for the assay of urinary heterophile haemagglutinins. A new finding in this study was the existence of high reacting and low reacting sheep erythrocytes. The isolation of this former group proved to be essential for the success of the renal studies.

Finally, these techniques were used to study urinary excretion of F.D.P. and heterophile haemagglutinins in glomerulonephritis and renal homotransplantation. These studies confirmed the concept that the origin of urinary F.D.P. in proliferative glomerulonephritis and renal transplantation is likely to be immune. But, that a group of patients can be demonstrated in the former conditon, which was hitherto unrecognised. It is concluded that the introduction of urinary heterophile haemagglutinins quantitation represents a new and valuable approach to further studies on the pathogenesis of glomerulonephritis

and renal homtransplantation rejection, and their response to therapy.

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APPENDIX

Preservation of Human O cells fixed with glutaradehyde

Materials :-

Glutaraldehyde - 25% in distilled water (Koch Light Lab. Ltd.)

Phosphate Saline Buffers - pH 8.0 (P.B.S.)

1 volume of 0.15M Na_2HPO4 (Anhydrous)

9 volumes of 0.15M NaCl

5 volumes of distilled water - pH adjusted to 8.0 with N/1 HCl.

Saline Solution - 0.15M NaCl in distilled water.

Sodium Azide - (British Drug House Ltd.)

100 mg/ml in distilled water.

Preparation of glutaraldehyde mixture - 1% glutaraldehyde in P.B.S. was prepared by adding 4 parts of 25% glutaraldehyde to 96 parts of P.B.S. (V/V) and the mixture chilled by immersion in iced water until use.

Preservation of Human O cells fixed with formaldehyde by three different methods

Materials :-

Formaldehyde - 40% B.P. (Evans Medical Ltd.)

Phosphate Buffered Saline - pH 7.2

761 ml of 0.15M Na_2HPO4 (Anhydrous)

239 ml of 0.15M KH_2PO4

1000 ml of 0.15M NaCl

The pH was adjusted to 7.2 with N/1 HCl or NaOH.

The conditions adopted for formalization of human O cells by three different methods are described in Table XXXVI

Preservation of Human O Cells fixed with Pyruvic aldehyde according to Ling (1961).

Materials :-

Pyruvicaldehyde - 40% (Sigma Chemicals Ltd.)

Phosphate Buffer - pH 8.0

945 ml of 0.15M $NaHPO_4$ (Anhydrous)

55 ml of 0.15M KH_2PO_4

pH adjusted to 8 with N/1 HCl

Treatment of Cells

1.5 vol. of a 25% pyruvic aldehyde solution (by adding distilled water to 40%) was brought to a pH of 7.0 by the addition of a 1% Sodium Carbonate Solution. 0.7 vol. of 0.15M phosphate buffer (pH 8.0) was added, followed by 1 vol. of 50% suspension of cells in Saline. The suspension was incubated at $4^{\circ}C$ for 24 hours with occasional mixing and then washed three times in Saline and a 10% suspension was made in Saline containing 0.1% sodium azide.

Tanning procedure :

Tannic Acid (May and Baker Ltd.)

A fresh stock solution of tannic acid in distilled water (10 mg/ml) was used, and working solutions of 1 in 40,000 made by further diluting in buffer (P.B.S.).

Procedure for coating cells with fibrinogen

Citrate Phosphate Buffer - pH 6.4 (C.P.)

Fixation of human O cells with formaldehyde
by three different methods

Conditons adopted for formalization	Methods used for formalization		
	Wide's (1962) modification of the method of Weinbach (1958)	Method described by Herbert (1967)	Method described by Ling (1961)
Temperature (^{o}C)	37	37	First step - 4 Second step - R.T. (22)
Formaldehyde Conc. (%)	3 (40% B.P. taken as 100%)	1	First step - 30 Second step - 6
Buffer and pH	Saline or P.B.S. 7.2	P.B.S. 7.2	Saline
Time	18 - 22 hours	18 hours	First step - 2 days Second step - 2 days

TABLE XXXVI

Citrate Phosphate Buffer - pH 6.4 (C.P.)

350 ml of 0.15M Na_2HPO_4 (Anhydrous)

650 ml of 0.15M KH_2PO_4

1000 ml of 0.10M tri-sodium citrate

pH adjusted to 6.4 with 0.5N citric acid

Human Fibrinogen -(Kabi Pharmaceuticals Ltd. 97% clottable protein)

A freshly prepared stock of fibrinogen solution containing 20 mg/100 ml in distilled water was used, and working solutions of 10µg/ml were made in C.P. for coating of cells.

Diluting Fluid

A 2% solution of bovine albumin (Armour Pharmaceuticals Ltd.) in C.P. buffer pH 6.4 containing sodium azide (1 mg/ml) was used.

Preparation of test sera for F.D.P.

Trasylol (Aprotinin) - Bayer Pharmaceuticals Ltd.

5 ml vial containing 25000

Kallikrein Inactivator Units (K.I.U.)

Topical Thrombin -(Parke Davis & Co.)

5000 units per vial dissolved in physiological saline at a dilution of 100 units/ml and was stored at -40°C.

Preparation of test sera

10 mls of blood was withdrawn by clean venepuncture with minimal venous occlusion, using a disposable plastic syringe. The blood was transferred into a glass tube containing 0.2 ml of aprotinin (5000 K.I. units per ml). The mixture was incubated at 37°C for 4 hours. The upper two thirds of serum was separated after

Centrifugation for 10 minutes at 3400 R.P.M. Thrombin was then added to give a final concentration of 10 units per ml which was followed by a further 30 minutes incubation at 37°C and then centrifugation at 3400 R.P.M. for 5 minutes. The resultant serum was either used immediately or stored at -40°C.

Antibody titration :-

Special Apparatus

The microtitre system (Cooke Engineering Co., U.S.A.) was used throughout the performance of the test. The microtitre kit consists of permanent "Lucite V" plate, calibrated micro-pipette dropper, Takasy microtitrators and a reading mirror.

Materials :-

Tris Buffer - pH 7.8

0.15M solution of tris (hydroxymethyl) - amino methane (Koch-Light Laboratories) pH adjusted to 7.8 with N/1 HCl

Standard Fibrinogen - Human Fibrinogen (Kabi Pharmaceuticals Ltd.) at three concentrations 1.25, 2.5 and 5 mg/ml (W/V) in tris buffer pH 7.8 was incorporated in the test system. The amount of clottable protein in these standards was calculated by the method of Ratnoff and Menzies (1951) as modified by Alkjaersig (1960) and the mean value was taken.

A small aliquot of 0.4 ml was kept in a Teklab tube and stored at -40°C as a stock solution. The stock solution of each of the three concentrations was diluted 100 folds in diluting fluid before incorporated in the test. In the test the diluted fibrinogen was

further diluted along with the test samples.

Anti-human fibrinogen serum from rabbit - (Behringwerke AG, Batch No. 1154 P)

The neat antiserum was diluted 1 in 500 in diluting fluid (C.P.) and stored as aliquots of 0.5 ml at -40°C. Working solutions were made by thawing the aliquot and further diluting in diluting fluid. Monospecificity was determined by immunodiffusion and immuno-electrophoresis against human plasma and fibrinogen.

Antibody Titration

The antifibrinogen serum (which was diluted previously 1: 500 and kept as stock solution at -40°C), was thawed anddiluted further in diluting fluid (doubling dilutions) as follows:-

By using a micropippete dropper,one volume (0.025 ml) D.F. was placed in each well of a given row by omitting the first well. Two volumes (0.05 ml) of antisera (1: 500 dilutions) were then placed in the first well and the microdilutor was then inserted into it and then rotated 20 times. The microdilutor was then transferred to the next well of the row carrying along 0.025 ml of antisera and mixed by swirling the microdilutors 20 times to ensure good mixing and thus produced doubling dilution. It was then transferred into the next well and the same process was repeated, the result was 1: 4 dilution of the original antiserum. By repeating this process 8, 16, 32 and so on folds of dilutions of antiserum were obtained. One volume (0.025 ml) of diluting fluid and one volume (0.025 ml) of 2.5% suspension of fibrinogen sensitised cells were then added in each well containing the

diluted antisera. The contents of the wells were then mixed by tapping or shaking the plate. The plates were then covered with parafilm or by another plate to prevent evaporation and incubated at room temperature (22°C) at least for four hours or better, left overnight without disturbance. The results were noted by observing the settling pattern of the cells at the bottom of the wells with the help of a reading mirror.

Positive and negative control was incorporated as such -

positive control includes one drop of diluting fluid, one drop of antisera (stock solution of 1/500 dilutions) and one drop of a 2.5% suspension of sensitised cells.

Negative control constitutes two drops of diluting fluid and one drop of 2.5% suspension of sensitised cells.

Antibody Titre:-

The highest dilution which showed complete agglutination (+) was taken as the end point and called the antibody titre. The antibody titre was expressed as a reciprocal concentration of antiserum where complete agglutination occurred.

Patterns of Agglutination

Complete agglutination was designated as positive (+) where the cells form a smooth mat at the bottom of the well.

Incomplete or partial agglutination was designated as ($\pm$) where the cells partly formed a smooth mat around an indistinct dispersed button of cells.

No agglutination was designated as (-) where the cells settled as a firm distinct button at the centre of the wells.

Calculation of F.D.P.

The amount of F.D.P. present in an unknown sample is calculated from the inhibition titre obtained by a known amount of clottable protein present in the standard fibrinogen solution.

Therefore, the amount of F.D.P. in the unknown is calculated as follows :-

Fibrinogen/fibrin degradation products

= sensitivity of the standard x inhibition titre of the solution. test.

$$\text{Sensitivity} = \frac{\text{The quantity of clottable protein in standard fibrinogen}}{\text{The inhibition titre of standard fibrinogen}}$$

Materials for Fibrinogen Assay

Barbitone - Saline Buffer - pH 7.22

Sodium barbitone	-	5.71G
Sodium chloride	-	2.93G
Distilled water	-	960 ml

pH adjusted to 7.22 with N/1 HCl (approximately 25 mls) and the volume made up to 1 litre with distilled water.

Calcium - Thrombin

1 vol. of bovine thrombin (10 units/ml)

1 vol. of 1.12M calcium chloride ($CaCl_2$)

Mixed together and stored in 0.5 ml aliquots in a Teklab tube at $-40^{\circ}C$

Folin and Ciocalteu's Reagent (British Drug Houses Ltd.)

1 part of Folin Ciocalteu's Reagent was diluted with 2 parts of distilled water.

Solution of Epsilon Amino-caproic Acid (E.A.C.A.) in Saline

E.A.C.A.	-	710 mg
Physiological Saline (0.9%)	-	1,000 mls.

Fibrinogen Assay

Two methods were used.

a) Fibrinogen assay described by Ratnoff and Menzies (1951) as modified by Alkjaersig (1960).

This method was used as the standard reference with which other fibrinogen or its derivatives could be compared. In the test, the fibrinogen is clotted with thrombin in a medium containing sufficient E.A.C.A. to prevent proteolysis and incubated overnight. The resultant clot is precipitated, separated and hydrolysed with sodium hydroxide and boiling. The amount of tyrosine released by hydrolysis is estimated with Folin Ciocalteu's reagent and the fibrinogen concentration calculated from a standard tyrosine curve.

The tests were performed in triplicate in 5' x $\frac{5}{8}$" unsiliconised test tubes. The following reagents were added one after another - 6.0 ml E.A.C.A. Saline, 0.2 ml of 2.5% Calcium Chloride Solution, 0.1 ml of thrombin (100 units/ml in saline) and 0.2 ml of test solution and then mixed thoroughly. To ensure complete clot, the tubes were left overnight at 4^{o}C. The tubes were then centrifused at 3,400 R.P.M. at 4^{o}C for 5 minutes after addition of 0.5 ml of glass beads (diameter 0.15 mm) which aids the subsequent precipitation

of the fibrin. The supernatant was discarded and the glass beads and entrapped fibrin were washed 3 times with saline and then hydrolysed by the addition of 0.4 ml of 10% sodium hydroxide and boiled for 20 minutes in a water bath. The tubes were then cooled and 2 ml of 0.5N sodium hydroxide and 0.6 ml of Folin and Ciocalteu's reagent (diluted 1: 2 in distilled water) were added in sequence and mixed, and the tubes centrifuged at 3,400 R.P.M. at room temperature for 2 minutes. The tyrosine released by hydrolysis is assessed by measuring optical density in an Unicam S.P. 600 at 650 mμ using the red filter against the non-clotted tube as the blank. The tyrosine released is calculated from a previously prepared standard tyrosine curve, and converted to fibrinogen concentration by multiplying by 11.7. This technique is time consuming and tedious, and not easily adapted for routine work.

b) Fibrinogen assay as described by Ellis and Stransky (1961)

This provided a rapid reproducible assay, easy to perform and was used for routine purposes. In this study, the assay was performed for fibrinogen estimation in control fibrinogen as well on serial aliquots of an in vitro human fibrinogen plasmin digest. The optical density resulting from the formation of fibrin is measured in citrated plasma, as well test samples recalcified and clotted with thrombin. By comparison with a previously prepared standard curve, the fibrinogen content of the test can be calculated.

0.5 ml of test is added to 5.5 ml barbitone saline buffer (pH 7.22) in a glass test tube, mixed by inversion. 3.0 ml aliquots

of this solution are added to one unicam cells for the test and the other for the blank. 0.05 ml of calcium thrombin solution is then added to the test cell and mixed by inversion. After incubation at room temperature for 20 minutes, the optical density of the test cell is compared with the blank in an unicam S.P. 600 at 470 m with a blue filter.

Heterophile haemagglutinin in sera

Materials :

Phosphate Saline Buffer pH 6.4

350 ml of 0.15M $NaHPO_4$ (anhydrous)

650 ml of 0.15M KH_2PO_4

1000 ml of 0.15M NaCl
pH adjusted to 6.4 with N/1 HCl

Diluting fluid

Phosphate saline buffer pH 6.4

2% bovine albumin

0.1G% Na azide

Alsever's solution

Dextrose - 2.05G

tri-sodium citrate - 0.80G

sodium chloride - 0.42G

Distilled water - 100 ml

pH adjusted to 6.1 with 10% citric acid.

Autoclaving at 10 pounds for 10 minutes.

Sheep Cells

A panel of 10 animals, with a wide genetic variability, was selected from Marino and Finnish Landrace breeds. Selection was based on blood groups R, r', MK, LK and haemoglobin AA, BB and AB (Table XXXVII).

Details of the blood groups and breeds of

Sheep used for cell panel

Sheep Identification	Blood Groups	Breed
S11	r/AA/LK	Finnish Landrace
S12	R/AA/LK	Finnish Landrace
S17	r/AB/HK	Finnish Landrace
S18	r/AA/HK	Finnish Landrace
S19	R/AA/HK	Finnish Landrace
S24	R/AB/LK	Merino
S25	r/AB/LK	Merino
S26	R/BB/HK	Merino
S27	r/BB/HK	Finnish Landrace
S28	R/AB/HK	Merino

TABLE XXXVII

Jugular vein blood was withdrawn into Alsever's solution (1 part + 1 part) stored at 4°C for 3-4 days and preserved in formaldehyde followed by treatment with tannic acid (Das, 1970a). See Table XXXVI

Heterophile haemagglutinin in urine

Erythrocytes from three different species in high and low reacting sheep, white New Zealand rabbit and Sprague Dawley rat, were used to quantitate the heterophile haemagglutinin in the urine. Sheep blood was collected by venepuncture of the jugular vein, rabbit and rat blood by cardiac puncture and then mixed in Alsever's solution (1 part blood and 1 part Alsever's solution) and stored at 4°C for 3 - 4 days. The cells were presevered in glutaraldehyde as described for human O cells (vide supra). The fixed cells were suspended as 10% in distilled water containing sodium azide (1mg/ml) and stored at 4°C unitil use.

Performance of the test

Microtitre kit (Cooke Engineering Co., U.S.A.)

Diluant - distilled water - 1000 ml

sodium azide - 1G

The concentrated urine was prepared as described (vide supra) and was centrifused at 3,400 R.P.M. for 30 minutes. The clear supernatant was separated as aliquots in Teklab tubes. One drop of diluent was placed in each well of a plate except the first, where two drops of test urine was added.

Three controls were included in the test, i.e. pooled normal sera, normal urine and negative control. After titration of the test, as described above, One drop of diluent and one drop of

2.5% suspension of fixed erythrocytes were placed in each well (according to the designated wells for different species erythrocytes) and the mixture in the wells was mixed by shaking the plate. The plates were then incubated overnight at 4°C, and read on the following morning. The arbitrary score was given according to the pattern of agglutination as described (vide supra).

Serial concentrated urines (9 samples) from a transplant patient with a rejection episode, were tested for heterophile haemagglutinin against glutaraldehyde fixed sheep (Fig. 28) and rabbit (Fig. 29) red cells. One to three rows prerejection and 4 - 9 rows rejection samples, the 10th row is normal urine, 11th row is controlled normal sera and the 12th row is negative control. The figure also shows that the rabbit erythrocytes detect heterophile haemagglutinin in urine sample 4 more strongly then the sheep cells and gave a higher haemagglutinin score than sheep cells in sample 9. The pattern of agglutination can be seen in Figures and their score in Table XXXVIII.

Paul-Bunnel Test with Differential Absorption

The method of Barrett (1941) with some modification was used to determine whether the reaction is a Paul-Bunnel type, i.e. that it is absorbed by Ox cells but not by guinea-pig kidney.

Reagents: 1) Two mls of concentrated urine or serum previously inactivated by heating at 56°C for 30 minutes.

2) A 20% autoclaved Ox red-cell suspension

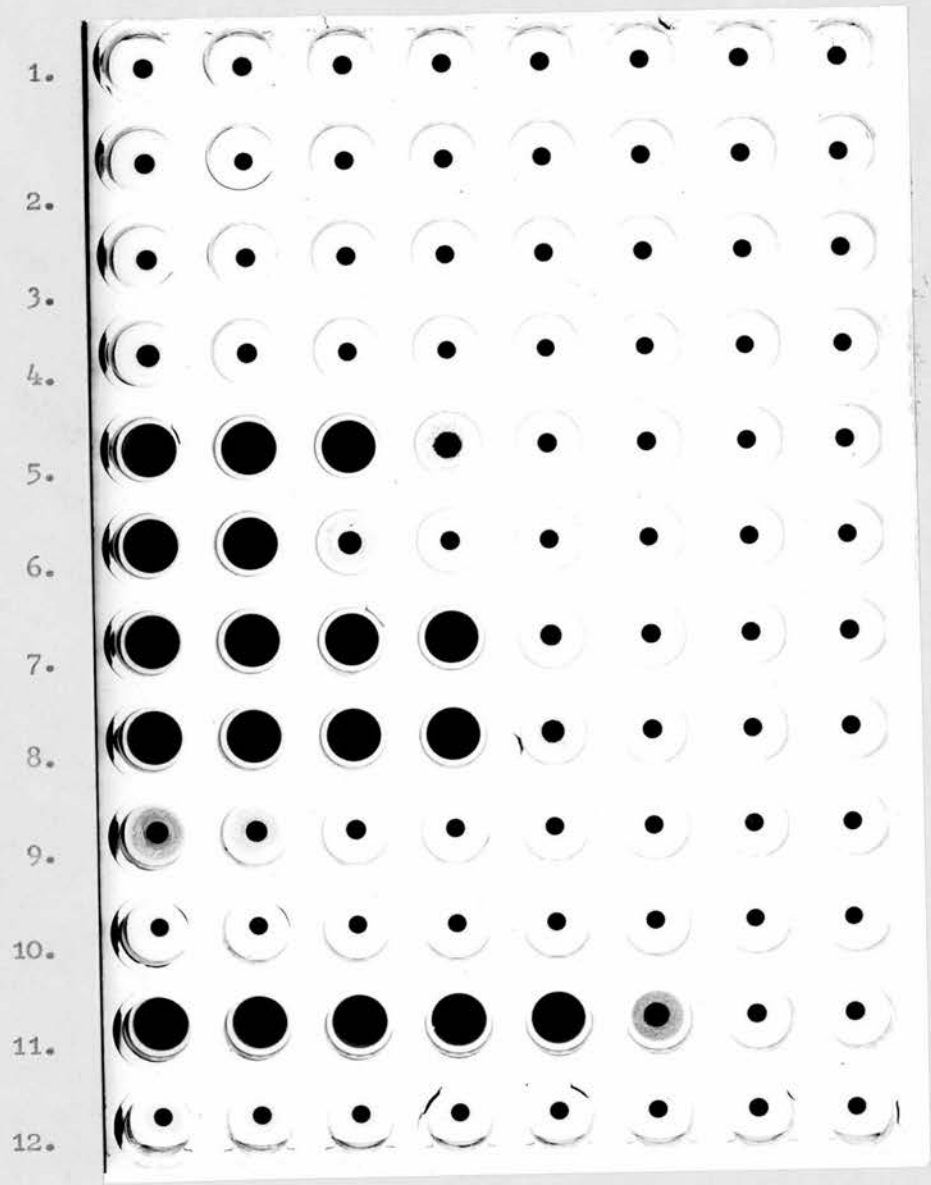

Fig. 28 Microtitre plate showing haemagglutination pattern of urinary heterophile (sheep) haemagglutinin. Rows 1 - 9 are urine samples from a transplant patient with a rejection episode, (rows 1 - 3prerejection and 4 - 9 rows rejection samples). The tenth row is normal urine, whereas the eleventh row is a control serum and the twelfth row a negative control.

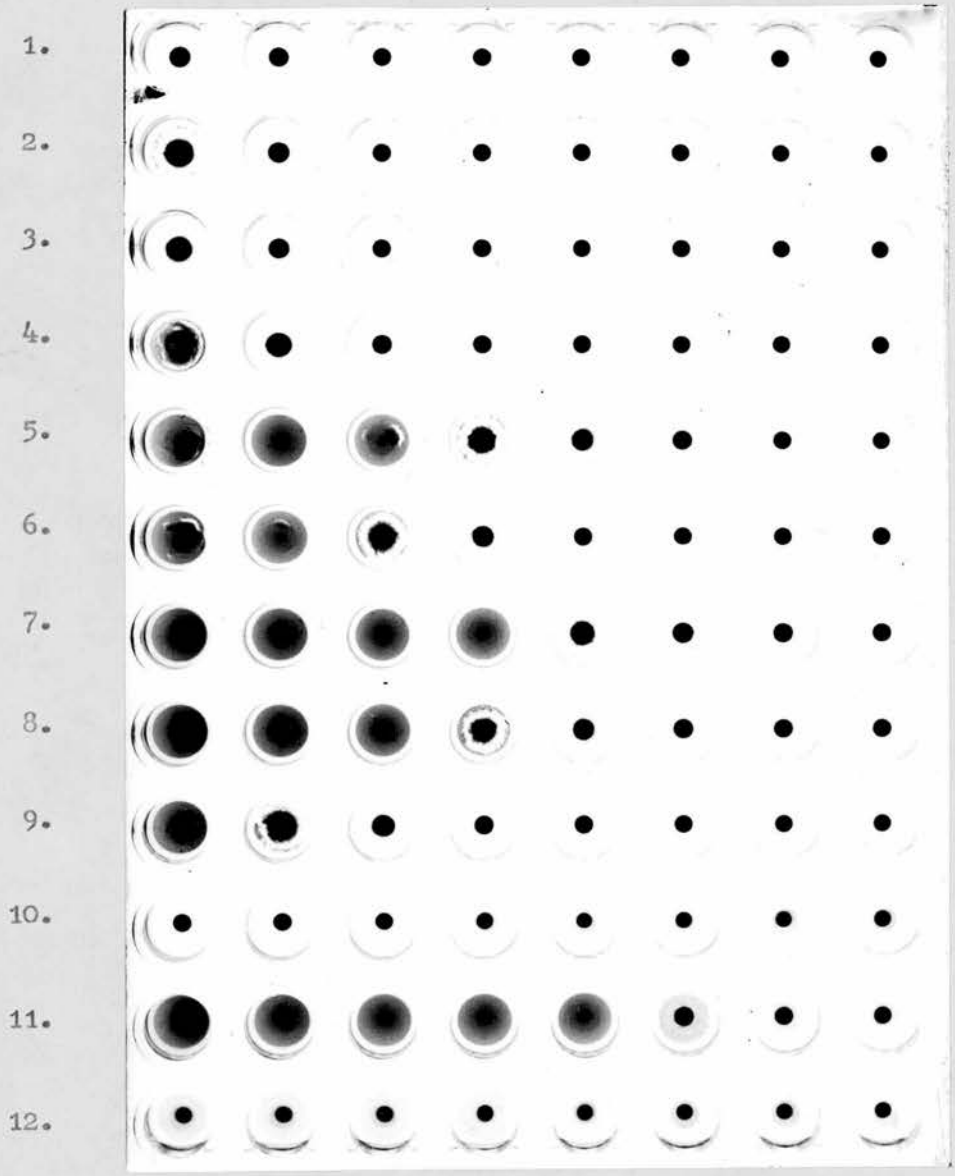

Fig. 29 Microtitre plate showing haemagglutination pattern by urinary heterophile (rabbit) haemagglutinin. 1 - 9 rows are urine samples from a transplant patient with a rejection episode, (1 - 3 rows are prerejection and 4 - 9 rows are rejection samples). The tenth row is a normal urine, the eleventh row a control serum and the twelfth row a negative control. (Note rabbit erythrocytes have detected agglutinins in higher proportion of the tested samples and have given overall higher titre than sheep).

Haemagglutination Score of serial urine samples from a transplant patient with rejection episode.

Test Sample	Sheep	Rabbit
1. Urine from a transplant patient	0	0
2. " "	0	2
3. " "	0	2
4. " "	2	7
5. " "	18	20
6. " "	12	17
7. " "	22	24
8. " "	22	22
9. " "	5	12
10. (Normal Urine)	0	0
11. (Normal Controlled sera)	28	28
12. (Negative Control)	0	0

* Rejection diagnosed clinically on day 4.11.71 (sample number 4.)

TABLE XXXVIII

3) A 20% guinea-pig kidney emulsion.

Absorption procedure

Three 0.5 ml volumes of test sample are delivered into three small tubes designated as A, B and C. 0.6 ml of guinea-pig kidney emulsion is added to tube B, 0.6 ml of ox-red cells to tube C and 0.5 ml of saline to tube A. The contents of the tubes are then mixed and incubated overnight at 4°C. The tubes are then centrifused and the supernatant retained. In the absorption procedure, the test samples become diluted 1: 2. The absorbed and unabsorbed test samples are then run for heterophile haemagglutination test using 2.5% glutaraldehyde fixed sheep cells (vide supra).

Thrombin time as described by Thomson (1970) with some modification

This method was used to measure the anticoagulant activity of in vitro digested products of fibrinogen.

Materials :-

Sodium citrate B.P. 3.8% (Boots Pure Drug Co. Ltd.)

Thrombin (Bovine thrombin - 10 units/ml in saline).

Citrated normal plasma - by clean venepuncture, 10 mls blood was withdrawn, placed in citrated tubes (9 parts blood to 1 part sodium citrate) and thoroughly mixed. Plasma was separated by centrifugation at 3,400 R.P.M. for 15 minutes.

Method :-

Test material (0.1 ml) and citrated normal plasma (0.1 ml) were placed in a glass tube and incubated for 1 - 3 minutes in an automatic coagulometer thermostatically controlled to 37°C. The

clotting time was initiated by adding 0.1 ml of iced cold thrombin and the subsequent clotting time noted and compared with the clotting time of a control in which saline 0.1 ml substituted for the test material. The test was always done in triplicate and the mean was taken.

Materials for Ouchterlony immunodiffusion plate or immunoelectrophoresis.

1. Barbitone - acetate buffer pH 8.6 and ionic strength 0.1M.

   Sodium barbitone - 50G

   Sodium acetate - 50G

   N/HCl - 32 ml (approximately)

   Sodium barbitone and sodium acetate (trihydric) was dissolved in 4L distilled water and the pH was adjusted to 8.6 using N/1 HCl and the final volume would be 5 litres by adding distilled water.

2. Agar (Difco)

   1.5G agar and 0.1G sodium azide was mixed to a solution of 50 mls distilled and 50 mls barbitone acetate buffer (0.1M). The mixture was then heated in a boiling waterbath until the agar dissolved and gave a clear solution. The temperature was then brought down to 65°C and then used either to prepare Ouchterlony plate or immunoelectrophoresis glass slides.

3. Ponceau S solution

   0.2% ponceua S stain in 6% sulpho sali cylic acid.

Ouchterlony immunodiffusion technique (Ouchterlony, 1962)

Onchterlony plates were prepared by pouring 15 mls of 1.5% melted agar into 8.8 cm Petridishes and allowed the gel to solidify for 4 hours. The wells - one central and six peripheral were cut by using a commercially available punch antiserum was placed in the central well and test materials in the peripheral wells and allowed to diffuse for 48 hours at room temperature (22°C) in a moist chamber.

Immunoelectrophoresis

The method described by Scheidigger (1955) was used. Glass slides (2.5 cm x 7.6 cm) were placed in a commercially available slat and 10 mls of 1.5% melted agar was poured in each portion of the slat (2 ml for each slide) and allowed to solidify for 4 hours. Wells for antigen and troughs for antiserum were cut in each slide by using a commercially available punch. Electrophoresis was carried out by using barbitone acetate buffer (pH 8.6 ionic strength 0.1M) inside the tank and electrophoresed for 2 hours by passing a current at the rate of 7.5 m A per section or 15 m A per slat followed by immunodiffusion for 48 hours in a moist chamber. The slides then covered with azide saline for a further 24-48 hours and then dried with filter paper and washed with tap-water. The slides were then stained in Ponceau S for 10 minutes and washed in 2% acetic acid until all excess stain was removed and then dried in air.

Urinary Protein Estimations

The buiret method of Hiller et al (1948) was used for estimation

of the protein in urine (unconcentrated) as per cent. Urinary protein is first precipitated with an equal volume of 10% (W/V) trichlor acetic acid, the precipitate dissolved in 30% (W/V) NaOH and the buiret colour developed by 5% (W/V) CuSO4. The concentration of protein is determined by a Lovibond Comparator. The protein estimation was performed in the Department of Clinical Chemistry, University of Edinburgh.

The Mancini method for quantitation of urinary IgG, IgM, C-3 and α 2 macroglobulin.

The IgG, IgM, C-3 and α2 macroglobulin content of concentrated urine, were quantitised by the Mancini technique (1965) using commercially obtained Hyland plates.

Priniple :- Antigen placed in a well in antibody containing agargel, diffuses into the gel and forms a precipitin ring. The total diameter of this ring is related to the concentration of antigen. Each immunodiffusion plate contains 12 wells, three of them required for three reference sera which are provided with the test kit. Thus one plate is used to prepare a standard curve and simultaneously to test 9 unknown specimens. As urine contains less antigen than serum, the reference serum provided with lowest quantity of antigen, was further diluted to get a curve, which could be fitted with a less amount of antigen.

Materials :-

1) Immunodiffusion plates (Hyland) containing antiserum specific for different antigen (IgG, IgM, C3 and α2 Macroglobulin).

11) Capillary pipettes (3.550" x 0.035" - Hyland)

111) Reference standards (Hyland), the lowest standard serum was

further diluted with saline (0.9%).

Performance of the test

The plastic cover of the immunodiffusion plate is removed and allowed to dry for 30 minutes if the plates contain moisture. The well is filled with the test specimen or reference serum by touching the tip of the capillary tube to the bottom of the well and allowed to flow by gravity so that the well is filled to the level of agar surface by taking precaution of overfilling or underfilling. The plate cover is then placed and the plates designated for IgG is incubated for 4 hours at 37°C and the other plates at room temperature (22°C) in a moist chamber for 24 - 48 hours.

The diameter of the precipitin ring is measured by using an eyepiece reticle having a mm. scale. The concentration of antigen in unknown specimens are calculated from the reference curve.

STATISTICAL ANALYSIS

Analysis of results for Student's "t" test, coefficient of linear correlation and rate of regression and standard deviation were obtained using the Olivetti programma 101 with the following equations :-

1. Student's "t" test

(a) Comparison of means of two samples:

$$M_1 = \frac{\Sigma x}{N_1} \qquad M_2 = \frac{\Sigma x}{N_2}$$

$$s_1^2 = \frac{N_1 \Sigma x^2 - (\Sigma x)^2}{N_1 (N_1 - 1)} \qquad s_2^2 = \frac{N_2 \Sigma y^2 - (\Sigma y)^2}{N_2 (N_2 - 1)}$$

$$\sigma \sqrt{\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2}}$$

$$t = \frac{M_1 - M_2}{\sigma \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Where M_1 is the arithmetic mean of the first sample

M_2 is the arithmetic mean of the second sample

s_1 is the variance of the first sample

s^2_2 is the variance of the second sample

N_1 is the number of values in the first sample

N_2 is the number of values in the second sample

x and y are the variate values.

(b) Paired comparison.

$$t = \frac{\bar{d} - 0}{\sqrt{\frac{SD^2}{N-1}}}$$

Where $\bar{d}$ is the arithmetic mean of the difference of pairs

N is the number of pairs of observations

SD^2 is the sample variance.

2. Coefficient of linear correlation and rate of regression

The Bravais-Pearson coefficient of linear correlation.

Formula:-

$$r = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{\left[N\Sigma x^2 - (\Sigma x)^2\right]\left[N\Sigma y^2 - (\Sigma y)^2\right]}}$$

Where x, y = variables

N = number of observations.

The regression line between two variables x and y according to the equation :-

$$y = a + bx$$

Formulae:-

$$b = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N\Sigma x^2 - (\Sigma x)^2}$$

$$a = \frac{\Sigma x - b\Sigma x}{N}$$

Where x, y = variables

N = number of observations.

3. Standard deviation.

Formula used:-

$$\sigma = \sqrt{\frac{N\Sigma x^2 - (\Sigma x)^2}{N(N-1)}}$$

Where x = variate value

N = number of values.

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13. PREPARATION OF HUMAN CELLS FOR THE ASSAY OF SERUM FIBRINOGEN DEGRADATION PRODUCTS USING HAEMAGGLUTINATION INHIBITION

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Boyden's (1951) original technique in which tanned red cells are coupled with an antigen such as fibrinogen, can be adopted as an agglutination inhibition reaction, using antifibrinogen serum, for detection of circulating fibrin/fibrinogen degradation products (F.D.P.) (Merskey et al. 1966 ; Das 1970a ; Das 1970b). Although sheep red cells have been used in many laboratories with satisfactory results, difficulties have been encountered in the complete absorption of heterophile antibody which is a necessary and time-consuming procedure before every assay. This antibody is present in over 90 % of the normal population (Salo, 1966) and its titre increases markedly following organ transplantation (Rapaport et al. 1968) and severe burns (Kano et al. 1967).

The following communication describes the results of a study designed to circumnavigate this problem by developing tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) using human group O red Rh. negative cells. In particular, efforts were made to find a substitute for formaldehyde as human red cells display a marked tendency to autoagglutinate when preserved in this material.

Materials

Glutaraldehyde
– 25 % (Koch Light Lab. Ltd.)

Formaldehyde
– 40 % B.P. (Evans Medical Ltd.)

Pyruvicaldehyde
– 40 % (Sigma Chemicals Ltd.)

Sodium Carbonate
– (British Drug House Ltd.)

Phosphate Saline Buffer
– pH 8.0 (P.B.S.)
1 volume of 0.15 M Na_2HPO_4 (Anhydrous)
9 volumes of 0.15 M NaCl
5 volumes of distilled water. pH adjusted to pH 8.0 with N/1 HCl

Saline Solution
– 0.15 M NaCl in distilled water

Citrate Phosphate Buffer
– pH 6.4 (C.P.)
350 ml. of 0.15 M Na_2HPO_4 (Anhydrous)
650 ml. of 0.15 M KH_2PO_4
1000 ml. of 0.10 M tri-sodium citrate
pH adjusted to 6.4 with 0.5N citric acid.

Tannic Acid (May & Baker Ltd.)
A stock solution of tannic acid in distilled water (10 mg/ml) was used, and working solutions of 1 in 40,000 made by further diluting in buffer (P.B.S.).

Sodium Azide (British Drug House Ltd.)
100 mg/ml in distilled water.

Diluting Fluid
A 2% solution of albumin (Armour Pharmaceuticals Ltd.) in C.P. buffer pH 6.4 containing

sodium azide (1mg/ml.) was used.

Human Fibrinogen (Kabi Pharmaceuticals Ltd. 97% clottable protein)

A stock fibrinogen solution containing 20 mg/100 ml. in distilled water was used, and working solutions of 10 μg/ml. were prepared in C.P. pH 6.4.

Anti Human Fibrinogen Serum from Rabbit (Behringwerke Ag.)

The neat anti-serum was diluted 1 in 500 in diluting fluid and stored in 0.5 ml. aliquots at −20°C. Working solutions were made by further dilution in diluting fluid.

Methods

Red Cell Fixation Procedures

Blood was collected in acid citrate dextrose (4 parts to 1 part) and allowed to settle by gravity for 2-5 days. The supernatant plasma was removed and the cells washed 3 times in 50 volumes of saline. After the final wash, the packed cell volume was measured following centrifigation at 1000g for 5 minutes and the cells were chilled by immersion in iced water. Satisfactory formaldehyde fixation was attempted using 3 different methods :a Wide's (1962) modification of the method of Weinbach, and the techniques described by Ling (1961) and Herbert (1967). Pyruvicaldehyde fixation was done according to Ling (1961). Gluteraldehyde fixation was done as follows : a 2% red cell suspension was prepared in 1% gluteraldehyde in P.B.S., mixed well and incubated for 30 minutes at 4°C with occasional further mixing. The cells were then washed 3 times in 50 volumes of saline followed by 3 washes in 50 volumes of distilled water. After the final wash a 10% (V/V) was prepared in distilled water containing sodium azide (1mg/ml.) and stored at 4°C until further use.

Tanning Procedure

All types of fixed cells were washed 3 times in 50 volumes of P.B.S., the haematocrit established and a 2% suspension (V/V) prepared in P.B.S. One volume of this cell suspension was then mixed with 1 volume of 1 in 40,000 tannic acid and incubated for 30 minutes at 56°C with occasional mixing. Following incubation the cells were washed 3 times in 50 volumes of P.B.S.

Sensitization Procedure

All types of tanned and fixed cells were washed 3 times in 50 volumes of C.P. buffer and after the haematocrit was established a 4% suspension (V/V) was prepared in C.P. buffer. One volume of cells and 1 volume of a solution of human fibrinogen (10 μg clottable protein/ml. in C.P. buffer) were mixed and incubated for 30 minutes at 37°C. The sensitized cells were then washed 3 times in 50 volumes of C.P. buffer and finally made up as a 10% suspension (V/V) in diluting fluid and stored at 4°C. Three days later the supernatant fluid was replaced by an equal volume of fresh diluting fluid.

Serum F.D.P. Immunoassay

The details of the T.R.C.H.I.I. employed in this study have been described in detail elsewhere (Das, 1970b), with the exception that the microtitre plates were incubated at room temperature (20°C) and 2% bovine albumin was used as a stabiliser in the diluting fluid. The behavior of the cells in the capillary F.D.P. technique described by Israel et al. (1968) was also studied.

Results

Five batches of formaldehyde, 4 batches of pyruvicaldehyde and 17 batches of gluteraldehyde cells were prepared on separate occasions, tanned, sensitized at the same time under identical conditions, and their sensitivity to anti-fibrinogen sera ascertained along with negative controls in which diluting fluid replaced antisera. The results are summarised in Table I, which shows that gluteraldehyde fixation alone provided batches of cells with no trace of autoagglutination. Moreover, those cells fixed with formaldehyde and pyruvicaldehyde which appeared satisfactory on initial testing, developed autoagglutination within a month of storage at 4°C. Gluteraldehyde fixed cells, however, remained in excellent condition for more than 6 months, and the sensitivity of all batches to antibody was in excess of one part

Table I

Antibody titre and incidence of autoagglutination in batches of human group O cells fixed with a different agent and subsequently tanned and sensitized with human fibrinogen.

No. of batches	Type of Aldehyde	Antibody Titre	No. of batches with Autoagglutination in negative controls
5	Formaldehyde	$\frac{1}{1024\times10^3} - \frac{1}{2048\times10^3}$	3
4	Pyruvicaldehyde	$\frac{1}{256\times10^3} - \frac{1}{512\times10^3}$	2
17	Gluteraldehyde	$\frac{1}{1024\times10^3} - \frac{1}{2048\times10^3}$	0

Table II

Effect of variations in fibrinogen concentration, pH, time of incubation and temperature on the antibody titre, during the sensitization procedure.

Fibrinogen Conc. (μg/ml)	pH	Temp. (0° C)	Time (mins.)	Cell conc. (% V/V)	Antibody Titre	Autoagglutination in controls
			Fibrinogen Concentration			
1	6.4	37	30	4	$1/512\times10^3$	–
10	6.4	37	30	4	$1/2048\times10^3$	–
100	6.4	37	30	4	–	+
			pH			
10	6.4	37	30	4	$1/2048\times10^3$	–
10	8.0	37	30	4	$1/2048\times10^3$	–
			Time of Incubation			
10	6.4	37	15	4	$1/2048\times10^3$	–
10	6.4	37	60	4	$1/2048\times10^3$	–
			Temperature			
10	6.4	22	30	4	$1/2048\times10^3$	–
10	6.4	37	30	4	$1/2048\times10^3$	–

per million at initial testing and 6 months later.

Using gluteraldehyde fixed human group 0 cells optimal conditions for subsequent tanning and sensitization were also studied. Satisfactory tanning was achieved with either 20,000 or 40,000 tannic acid, the pH could vary between 6.4-8.0, the temperature between 22-56°C and the duration of tannic acid incubation could be as short as 15 minutes. There was a similar latitude in the conditions for sensitization, the results of which are shown in table II. However, a fibrinogen concentration of 100 μg/ml resulted in autoagglutination of the controls, but, high fibrinogen concentrations could be used if the cell concentration was proportionally increased. Thus a 16% (V/V) suspension was satisfactorily sensitized by 40 μg/ml of fibrinogen.

Further studies revealed that when using gluteraldehyde it was possible to exclude the tanning stage and sensitize the cells immediately after fixation. This was best achieved by fixation at 37°C and incubating a 4% cells suspension in a fibrinogen solution (30 μg/ml) at pH 6.4, for 60 minutes at 37°C. These cells gave a consistent antibody titre up to 1/64,000 and remained stable for more than 3 months.

In the serum F.D.P. assay the human group 0 gluteraldehyde fixed and tanned cells behaved in a similar manner to sheep cells with reference to reaction patterns and sensitivity to test sera. However, prior absorption of the unknown sera was not necessary, although it was essential to perform the test at room temperature in order to avoid interference from cold agglutinins. These cells also reacted satisfactorily in the rapid capillary technique described by Israels et al. (1968) as is shown in figure 1.

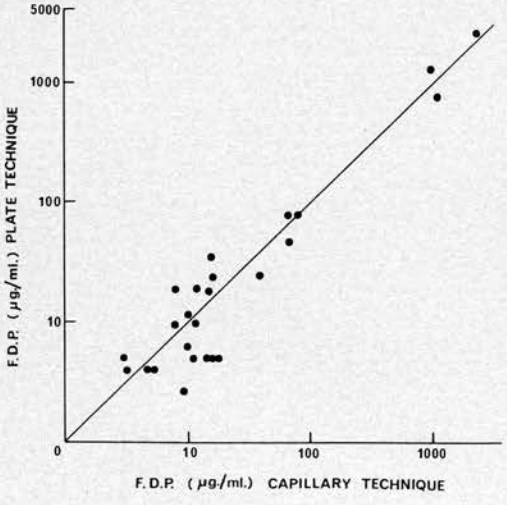

Figure 1

Comparative F.D.P. values of 25 serum samples assayed by the microcapillary and microtitre techniques.

Comments

Although a change from preserved sheep to human group 0 cells in the T.R.C.H.I.I. for the quantitation of F.D.P. would avoid the time-consuming problem of prior absorption of test sera, no reports have appeared describing the preparation and use of such cells. Merskey et al. (1969) reported their successful experiences with human red cells but these were not fixed and could not therefore be readily stored for prolonged periods of time.

The results of the present study have shown that whereas formaldehyde is a satisfactory fixing reagent for sheep red cells it is totally inadequate for human cells, as a high proportion of batches autogglutinate immediately after preparation and those which appear satisfactory deteriorate to such an extent that they are unusable within 1 month. The problem has been solved by introducing gluteraldehyde fixation. Cells prepared by this method are stable and sensitive and indeed direct sensitization is possible without tanning. These findings are similar to those of Bing et al. (1967) who studied the coating of sheep cells with proteins other than fibrinogen. Although the direct procedure produces cells which have a markedly lowered antibody sensitivity, they can readily be used in routine haemagglutination inhibition assays for serum F.D.P.

While rapid results can be achieved using unfixed human group O cells in a macrovolume system (Merskey et al. 1969) the cells for this modification cannot be stored for prolonged

periods. This is a significant handicap for those laboratories not engaged in research exercises but requiring rapid serum F.D.P. assays at infrequent intervals. The gluteraldehyde fixed human group O red cells which can be stored for many months, used in the capillary technique will answer this practical need.

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2

Variability of Sheep Red Cells in their Reaction to Agglutinins in Normal Human Sera

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Variability of Sheep Red Cells in their Reaction to Agglutinins in Normal Human Sera

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Summary. The interaction (agglutination) of 25 normal sera against a panel of 10 different sheep red cells was studied. There were highly significant differences in the agglutinability of different sheep cells to any one normal serum, and the cells could be divided into 'high reactors' and 'low reactors'. Absorption studies confirmed these observations. The significance of these findings is discussed in the light of previous studies on anti-sheep cell haemagglutinins in disease and the selection of sheep cells for all types of haemagglutinin immunoassays.

The introduction of specific, sensitive and semiquantitative haemagglutination inhibition immunoassays for a variety of proteins, using sheep red cells previously treated with tannic acid (Boyden, 1951), has been widely adopted, with varying degrees of success (Herbert, 1967). In using this technique for the assay of serum fibrin/fibrinogen degradation products (FDP), difficulties have arisen, from time to time, with the complete absorption of the natural anti-sheep agglutinins, prior to running the test serum in the immunoassay (Cash *et al*, 1969; Mertens *et al*, 1969). The following communication describes a series of studies in which it is shown that the capacity of red cells derived from different sheep to absorb the anti-sheep agglutinin in any one normal serum varied markedly.

MATERIALS AND METHODS

Sheep Cells

A panel of 10 animals, with a wide genetic variability, was selected from Merino and Finnish Landrace breeds. Selection was based on blood groups R, r; *HK*, *LK* and haemoglobin *AA*, *BB* and *AB* (Table I). Jugular vein blood was withdrawn into Alsever's solution stored at 4°C for 3–4 days and preserved in formaldehyde followed by treatment with tannic acid (Das, 1970).

Test Sera Preparation

The serum was prepared as for FDP estimations: 10 ml of blood was withdrawn into a glass tube containing 0.2 ml of aprotinin (5000 K.I. units per ml). The mixture was incubated at 37°C for 4 hr. Thrombin was then added to the separated serum, to give a final concentration of 10 units per ml, which was followed by a further 30 min incubation at 37°C and then centrifugation at 3400 rpm for 5 min. The resultant serum was either tested immediately or stored at −40°C.

TABLE I. Details of the blood groups and breeds of sheep used for cell panel

Sheep identification No.	*Blood groups*	*Breed*
S_{11}	r/AA/LK	Finnish Landrace
S_{12}	R/AA/LK	Finnish Landrace
S_{17}	r/AB/HK	Finnish Landrace
S_{18}	r/AA/HK	Finnish Landrace
S_{19}	R/AA/HK	Finnish Landrace
S_{24}	R/AB/LK	Merino
S_{25}	r/AB/LK	Merino
S_{26}	R/BB/LK	Merino
S_{27}	r/BB/HK	Finnish Landrace
S_{28}	R/AB/HK	Merino

Test System

The microtitre system (Cooke Engineering Co.) was used throughout, using 0.025 ml volumes. One volume of test serum was added, followed by 1 vol of diluting fluid and finally 1 vol of a 2.5% suspension of sheep red cells. The contents of the microtitre plates were mixed, incubated at 4°C for 16 hr and then read at room temperature. A system of scoring the agglutination titres arithmetically was used to summarize and analyse the data (Race & Sanger, 1954). A score of 5 was given to each serial dilution, thus agglutination end points at 1/1 scored 5, 1/2 = 10, 1/4 = 15, 1/8 = 20, 1/16 = 25 and 1/32 = 30 etc.

Absorption Procedure

Two volumes of test serum were added to 1 vol of washed packed red cells, mixed and incubated for 30 min at 4°C. After incubation the samples were centrifuged at 3400 rpm for 5 min at 4°C, separated and stored at −40°C until required.

RESULTS

Sera from 25 healthy volunteers were tested simultaneously against the panel of formalized tanned red cells. The results are shown in Table II. Whereas there was no significant difference between the mean titre scores of cells S_{11}, S_{12}, S_{17}, S_{18}, S_{19} and S_{27} (all Finnish Landrace), there was a highly significant lower reactivity between each member of this group and S_{24}, S_{25}, S_{26} ($P<0.001$) and S_{28} ($P<0.002$) (all Merino). Using formaldehyde preserved cells it was shown that these differences were highly reproducible, when the same sera stored at −40°C were retested some 6 mth later. Further analysis of the data revealed that, although the red cells from sheep S_{24}, S_{25} and S_{26} appeared to be 'overall' poor reactors, this did not apply to certain individual reactions. An example of this phenomenon is shown by the performance of serum R: S_{25} cells reacted more strongly than S_{11} cells, whereas S_{26} cells, which usually mirrored the reactions of S_{25} cells, failed to agglutinate.

TABLE II. Haemagglutination titres (expressed as scores) in 25 normal sera tested against a panel of 10 sheep cells

Sheep identification	*Sera identification*																							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*	*X*	*Y*
S_{11}	20	15	25	20	30	15	5	25	30	30	35	25	20	20	25	15	30	5	20	20	15	20	30	25	30
S_{12}	15	15	25	20	25	15	5	20	20	25	35	20	15	15	15	20	30	10	15	10	15	15	30	20	20
S_{17}	15	15	25	20	30	15	10	25	20	30	40	25	20	20	20	20	25	10	20	15	15	15	30	25	20
S_{18}	25	15	25	25	30	20	10	25	20	30	40	25	20	20	20	15	30	15	20	20	15	20	30	20	30
S_{19}	20	10	20	20	25	15	5	20	20	20	30	15	10	15	10	15	20	10	15	20	15	20	30	25	40
S_{24}	10	5	15	10	15	10	0	10	0	15	20	10	10	10	10	10	15	0	5	0	5	5	20	10	10
S_{25}	15	10	20	15	25	15	5	20	5	15	20	15	10	10	10	10	15	15	10	15	10	15	30	10	10
S_{26}	5	0	5	10	15	0	0	10	0	10	10	10	5	10	5	10	15	0	0	0	0	0	10	10	5
S_{27}	20	15	20	20	25	15	10	20	20	25	40	25	20	20	20	20	25	5	20	15	15	15	30	20	20
S_{28}	15	10	20	15	20	10	0	15	15	15	20	20	15	15	15	15	25	—	—	—	—	—	—	—	—

The basic concept of overall 'good' and 'poor' sheep cell reactors and the occasional individual exceptions was further substantiated by absorption studies. A single batch of cells tested 'fresh' and after tannic acid treatment against 50 normal sera demonstrated that tanned cells gave a significantly higher titre ($P < 0.001$).

DISCUSSION

Landois (1875) first recorded agglutination of red cells from different animal species by human sera. This work was initially extended by Landsteiner (1900), and subsequent studies have drawn particular attention to the occurrence of sheep cell haemagglutinins in normal human serum (Paul & Bunnell, 1932; Bunnell, 1933; Davidsohn, 1933; Stuart *et al*, 1935). The anti-sheep agglutinins in sera from patients with infectious mononucleosis (IM) and normal controls differ significantly. The antibody in IM is not absorbed by guinea-pig kidney (Davidsohn, 1937; Lippet & Nogalski, 1953) but is readily absorbed with boiled ox red cells (Kemp & Baker, 1936). The reverse is found in normal sera. Papain treatment of sheep cells increases the titre of anti-sheep agglutinins in normal sera, whereas it has little or no effect on the titre of IM sera (Wöllner, 1955; Salo, 1966). This latter property is perhaps analogous to our findings using tannic acid treated cells and similar to the observations of Munoz (1967) who studied the reactions of mouse sera on tanned sheep red cells.

An extensive search of the literature has failed to reveal previous reports on differences in reactivity between sheep cells to haemagglutinin(s) in *normal* human serum, although this phenomenon has been recorded in sera from patients with IM (Zarafonetis & Oster, 1950). Moreover, Cox & Vermillion (1956) suggested that the problem was sufficiently important to warrant preservation of sheep red cells in order to obtain standardized techniques for the serological diagnosis of IM.

Although the *2HK, AA* Finnish Landrace sheep gave titres significantly higher than the others, particularly the *2LK, AB* Merinos, and it is possible that the variation of agglutination titres may be partly explained by inherent genetic differences in the cell membrane, the sample size was not sufficient to justify a distinction being made between the effects of breed and blood group. It is also likely that an additional heterogenicity is created by variability in the strength of binding of different populations of anti-sheep cell haemagglutinins.

The practical significance of these results is of particular interest for it is suggested that sheep cells with a known low reactivity to anti-sheep cell haemagglutinins should be selected as the ideal inert carriers of antigen or antibody in all types of haemagglutination immunoassays. In doing so it may prove possible to abandon the time-consuming exercise of absorption prior to carrying out the immunoassay. In those circumstances where this does not prove possible, absorption should be done with known highly reacting cells. The data also suggest that the conflicting reports of the rise in titre of anti-sheep cell haemagglutinins associated with allograft rejection (Rapaport *et al*, 1968; O'Kane *et al*, 1969) may be explained on the differences in reactivity of the sheep cells used in the different centres. It seems likely that the introduction of a panel of sheep cells in future studies would be more revealing.

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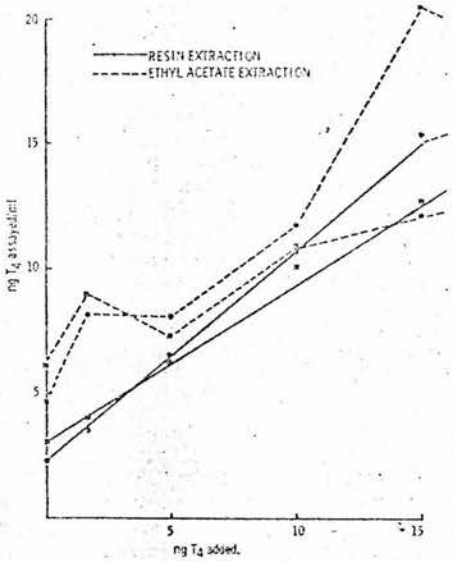

Comparison of resin extraction and ethyl-acetate extraction.

manifest when one compares the extraction procedures and recovery by assaying thyroxine after adding known amounts of the hormone to urine. If "assayable thyroxine" is plotted against the quantity of thyroxine added then the two should bear a linear relationship if the assay system is valid. If one considers the result of two such experiments (see accompanying figure) it can be seen that extraction procedures using ethyl acetate show a variable relationship to thyroxine added, whereas a linear relationship obtains with resin extracts. The plot does not pass through the origin because the urine already contains some endogenous thyroxine.

These results suggest that the resin-column procedure is to be preferred to ethyl-acetate extraction of urine before thyroxine estimation. Until further information is available, the term "urinary thyroxine" applied to the material assayed in these procedures is inexact and unjustified.

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THYROXINE IN THYROIDITIS

SIR,—We read with interest the article by Dr Papapetrou and his colleagues (Nov. 18, p. 1045). We agree that Hashimoto's thyroiditis, left untreated, will progress at different rates to result in hypothyroidism in the majority of patients.[1] Furthermore, we are not surprised that thyroxine failed to halt the advance of the underlying process. This we believe to be cell-mediated immunity.[2] Lamki et al.[3] from this laboratory have shown a significant inhibition of migration of peripheral leucocytes from patients with Hashimoto's thyroiditis when exposed to thyroid antigens. Moreover, we have found a significantly raised percentage of non-immune rosette-forming lymphocytes—i.e., T or thymus-dependent lymphocytes—in the peripheral blood of these patients.[4] No correlation was found between the percentage of T lymphocytes and the duration of the disease or between the percentage of T lymphocytes and titre of thyroid antibodies. It is believed that T lymphocytes mediate cellular immunity.[5] Laryea et al.[6] observed that lymphocytes from patients with Hashimoto's disease interfere with human thyroid-cell function in tissue-culture. Furthermore, Podleski[7] has shown that similar lymphocytes are capable of producing cytotoxic damage of cells coated with thyroid antigen. Antibodies to follicular cell components appear later through cooperation of thymic lymphocytes with bursa-equivalent (B) lymphocytes.

The fall in titre of thyroid antibodies recorded in children with lymphocytic thyroiditis treated with thyroxine[8] is probably coincidental to the spontaneous regression of the disease process, for it has been shown[9] that in a substantial proportion of affected children juvenile thyroiditis remits spontaneously.

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RAPID LATEX-SCREENING TEST FOR URINE F.D.P.

SIR,—Although our own experience with the rapid latex-screening test ('Thrombo-Wellcotest', Wellcome Reagents) for the semi-quantitation of urine fibrin/fibrinogen degradation products (F.D.P.) would support some of the specific findings of Dr Hulme and Mrs Pitcher (Jan. 6, p. 6), we are uncertain, at present, as to its future application.

In our experience approximately 50% of all concentrated and 15% of all unconcentrated urines (normal and pathological) contain non-specific latex agglutinins. This we have found can be reduced by freezing/thawing and centrifugation. However, there has remained a significant number which are resistant to this procedure and require either filtration through Whatman GF/B glass-fibre paper or prior absorption using latex particles coated with normal rabbit IgG. Thus, in order to be absolutely certain that the reaction is specific for F.D.P. we believe each urine should be set up against a control (latex coated with normal rabbit IgG). There is no doubt, however, that if these precautions are taken then the results of the Thrombo-Wellcotest correlate very closely with the hæmagglutination-inhibition immunoassay.[10]

Although it is probable that future studies may show that the rather aggressive approach of studying daily urine samples will not be necessary for all patients, it is likely that busy transplant units will still provide laboratories with substantial work-loads. In this context the microtitre system, used with the H.A.I., permits a significant degree of automation. If all the precautions necessary to avoid non-specific latex agglutination for the Thrombo-Wellcotest are instituted then the technique becomes exceedingly laborious and rather expensive. However, for a quick "one-off" test it could have a place, although the interpretation of a single observation may pose problems.

Finally, perhaps it should be pointed out that a urine concentration step may not be an essential feature of the H.A.I. technique. It was used in the original studies to ascertain whether excretion of urinary F.D.P. below 2·0 µg. per ml. was of clinical importance. This question has yet to be fully answered. The application of the Thrombo-

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Wellcotest, which has a lower sensitivity than the H.A.I., combined with the absence of concentration, could mean that some rejection episodes may be missed or detected rather late.

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LYMPHOCYTE-DEPENDENT ANTIBODIES

SIR,—Fakhri and Hobbs [1] demonstrated that antibodies and lymphocytes may cooperate in mediating tissue damage in patients with certain autoimmune diseases. They suggest that lymphocyte-dependent antibodies may be important in the pathogenesis of Hashimoto's thyroiditis, thyrotoxic exophthalmos, retinal vasculitis, and cœliac disease.

We have studied the serum of a patient with choriocarcinoma which contained a lymphocyte-dependent antibody (L.D.A.) specific for cells from the patient's husband.[2] This antibody has many similarities in common with the antibodies described by Fakhri and Hobbs. Using a ^{51}Cr-release assay, we found that neither the antibody alone nor lymphocytes alone were cytotoxic for target cells. However, the combination of L.D.A. and lymphocytes led to target-cell lysis. Neither conventional complement nor non-specific toxins were involved in L.D.A.-mediated cytotoxicity. Non-immune lymphocytes from man and other species could be recruited by L.D.A. to destroy specific target cells. Similar results have been reported in several animal model systems.[3–7]

Fakhri and Hobbs point to the need for further studies of the immunoglobulin class of L.D.A., types of inhibitors, and the nature of the cells participating in L.D.A.-mediated reactions. Recent studies indicate that L.D.A. migrates in the γ-globulin region during block electrophoresis and elutes in the 7S region of the G-200 'Sephadex' chromatographic column.[2,5] Goat antisera specific for IgG inhibits L.D.A. activity, whereas goat antisera specific for IgM does not inhibit L.D.A. activity. Studies in animals indicate that L.D.A. is detectable as early as 4 days after immunisation.[8] Lymphocytes (cells not adherent to plastic) but not monocytes (cells adherent to plastic) are capable of participating in L.D.A.-mediated reactions.[2] L.D.A.-mediated cytotoxicity appears to be mediated by bursa-derived lymphocytes.[9] The concentration of antibody coating target cells appears to be an important determinant of whether cytotoxicity or enhancement is produced.[10]

Complement-dependent antibodies have been known for many years to be important in the pathogenesis of hæmolytic anæmias and renal disease. Fakhri and Hobbs implicate lymphocyte-dependent antibodies in the pathogenesis of certain autoimmune diseases such as Hashimoto's thyroiditis, thyrotoxic exophthalmos, retinal vasculitis, and cœliac disease. Lymphocyte-dependent antibodies bridge the classical distinction between cellular and humoral immune responses. Cell-mediated tissue destruction may not be limited to cytotoxicity by sensitised T lymphocytes, but instead may include toxicity by a partnership of humoral, cell-dependent antibody, and non-immune host lymphoid cells. Early serum and cell transfer studies do not mitigate against this possibility, since immune sera could also contain blocking factors capable of shutting off cell-dependent antibody cytotoxicity in vivo. We agree with these workers that further studies of L.D.A. should be undertaken to better define their role in the pathogenesis of autoimmune diseases and their potential importance in the rejection of human cancers.

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ACUPUNCTURE ANÆSTHESIA

SIR,—Dr Karols (Dec. 30, p. 1417) questions the credibility of the reports on acupuncture. He states that "as long as the chest wall remains open there is no way that the patient can reinflate that lung through his own muscular efforts". Is it possible that the following explanation will do? The patient inspires on the intact side and then expires against closed upper airways (compare the Valsalva manœuvre). This will force the air from the intact lung over the bifurcation into the collapsed lung.

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FERRITIN IN SERUM

SIR,—The identification of β-fetoprotein as ferritin by Dr Alpert and his colleagues (Jan. 6, p. 43) is of considerable interest, but their suggestion that this protein appears in the circulation only under pathological conditions is not supported by our recent studies using a sensitive immunoradiometric assay.[1]

Ferritin is present in all normal sera and there is a close correlation between concentration of the protein and the iron stores of the body. The amounts found in different groups of subjects were [2]:

	No.	*Mean (μg. per l.)*	*Range*
Normal men	75	69·2	6–186
Normal women	44	34·8	3–162
Iron-deficiency anæmia	21	5·0	1–12
Transfusion siderosis	24	1790	170–5480
Idiopathic hæmochromatosis	8	2646	940–4240

In patients with refractory anæmia there is a close correlation between the serum ferritin concentration and the iron load from transfused red cells ($r = 0{\cdot}9$, $p < 0{\cdot}001$). In 22 normal subjects in whom iron stores were estimated by the phlebotomy technique [3] there is a similar relationship between serum-ferritin concentration and storage iron ($r = 0{\cdot}83$, $p < 0{\cdot}001$).

The occurrence of ferritinæmia in patients with liver disease or malignant states has long been recognised [4] and we have found the phenomenon in a variety of hæmatological malignancies. In 35 cases of acute myeloblastic leukæmia the mean serum concentration was 589 μg. per l. and in 19 cases of Hodgkin's disease 215 μg. per l.[5] Raised

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STUDIES ON A DIRECT LATEX AGGLUTINATION TECHNIQUE FOR THE SEMIQUANTITATION OF FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS.

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Presented at the conference on Fibrinogen and its derivatives,
Warsaw, October, 24th-26th 1972.

ABSTRACT

A simple, and reproducible direct latex agglutination technique is described, designed to quantitate F.D.P. The method appears to be satisfactory for rapid bedside estimations on serum, but its practical application for urinary F.D.P. measurements is limited.

INTRODUCTION

Over the last 10 years there has been a growing interest in the quantitation of fibrin/fibrinogen degradation products (F.D.P.) in serum and urine, on the basis that in some clinical situations elevated levels may represent direct evidence of fibrin deposition and its subsequent lysis (1). During this period there has been an associated development in technology to meet this need. Thus at the present time F.D.P. have been estimated by a variety of techniques both immunological (2,3,4) and non-immunological (5). All have there advantages and disadvantages but the demand for a simple, reliable, sensitive, specific and rapid one stage technique, using readily available reagents, has remained and its absence has partly been responsible for the retention of this technology within the bounds of highly specialised laboratories. In response to this demand a technique was introduced (known as the Fi test) in which latex particles, coated with antibody to human fibrinogen, agglutinated in the presence of fibrinogen or fibrinogen related antigen(s). In our own hands this reagent has been particularly unreliable and Thomas and his colleagues

(6) have considered that this feature may be due to the absence of reactivity to fragment E.

The recent introduction of an antibody-coated latex particle preparation coated with a mixture of specifically prepared anti-D and anti-E has raised the possibility of restudying this problem. The new latex reagent is known as the Thrombo-Wellcotest and we have compared its performance with the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.) on artificial digests of fibrinogen and clinical samples of urine and serum.

MATERIALS AND METHODS

Serum and urine test samples were prepared as described previously (7,8). All concentrated urine samples were centrifuged at 3,400 r.p.m. for 10 minutes at 4°C in a 6L M.S.E. Mistral centrifuge prior to assay. The T.R.C.H.I.I. was performed using the modifications of Das (9) of the Merskey technique (2). The Thrombo-Wellcotest reagent was kindly supplied by Wellcome Reagents Limited, Beckenham, Kent, England, as was a suspension of latex particles coated with normal rabbit IgG. Test samples and standards (human fibrinogen; Kabi Pharmaceuticals) were doubly diluted in a glycine buffer (0.1M in 1% NaCl containing 0.1% sodium azide at pH 8.2). Two drops (0.025 ml) of test material or standard were placed into a marked ring on a clean glass slide. Two drops of the latex suspension were then added, mixed using a glass rod and incubated at room temperature for 2 minutes. Macroscopic agglutination was visualised against a dark back-ground.

RESULTS

Sensitivity to fibrinogen, Fragments D and E

The direct latex test was shown to be sensitive to as little as 4 µg/ml of fibrinogen. Specific products (kindly supplied by Doctor V. Marder, Temple University, Philedelphia, U.S.A.) revealed a sensitivity to fragment D of 4 µg/ml and fragment E of 0.5 µg/ml.

Comparison of the T.R.C.H.I.I. and Latex Test during in vitro plasmin digestion of fibrinogen

Streptokinase (1000 units/ml; Hoechst Pharmaceuticals Ltd.) plasminogen (0.25 casein units/ml; Kabi Pharmaceuticals Ltd., Grade A) and human fibrinogen (10 mg/ml; Kabi Pharmaceuticals Ltd.) were incubated in Tris buffer at 37°C. 1 ml aliquots were removed at various intervals, the reaction stopped with

Trasylol (0.1 ml of 5000 units/ml; Bayer Pharmaceuticals Ltd.) and the thrombin time (10) a fibrinogen estimation (11), T.R.C.H.I.I. and Latex test performed.

The results are shown in Fig. 1.

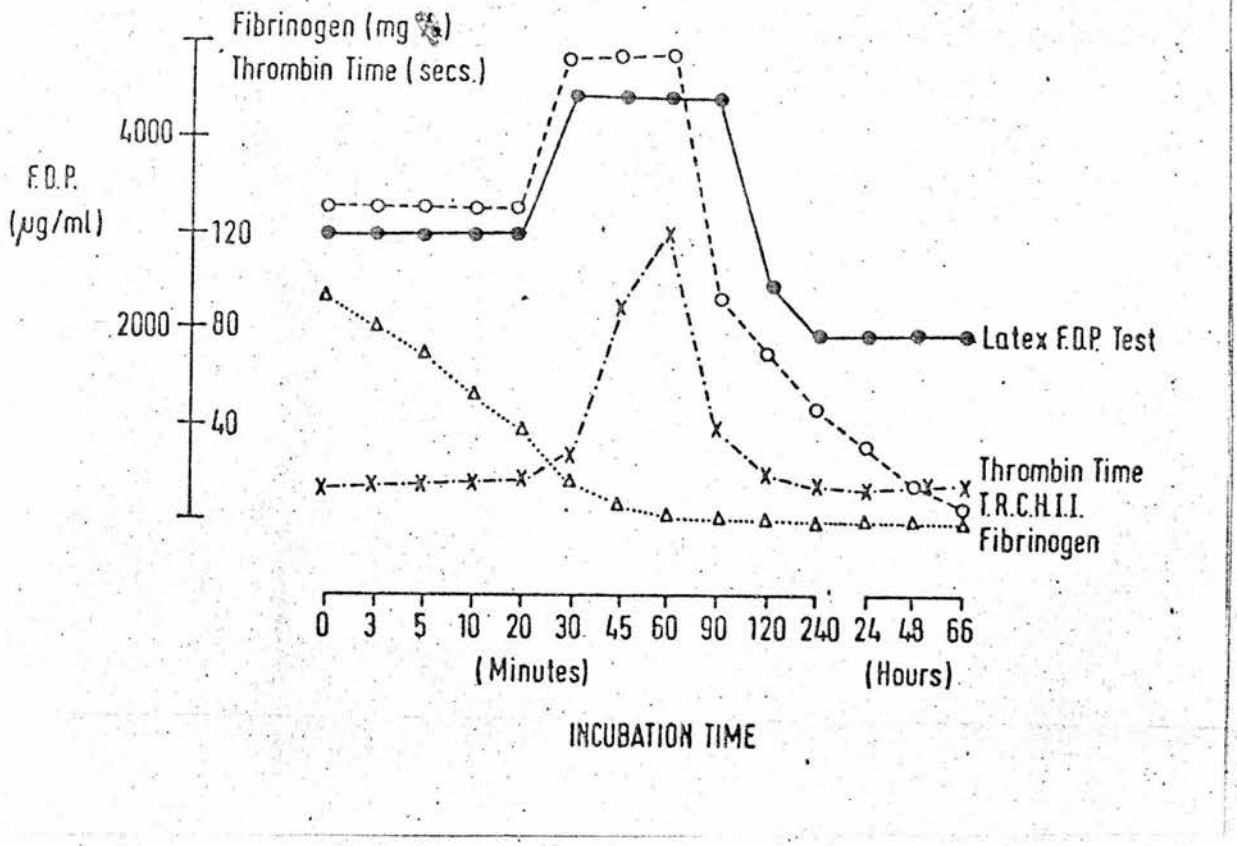

FIG. 1.

F.D.P. quantitation using the Latex Test and T.R.C.H.I.I. thrombin times and fibrinogen estimations on serial aliquots of an in vitro human fibrinogen plasmin digest.

The apparent higher sensitivity of the Latex test to later digests, compared to the T.R.C.H.I.I., was thought to be related to the use of antisera in the T.R.C.H.I.I. directed against whole fibrinogen, thus being relatively insensitive to anti-E. This conclusion was supported by the known high sensitivity to the E fragment of the Latex test and the observation that when a specific anti-E serum was used in the T.R.C.H.I.I. then continued sensitivity was retained throughout the period of digestion (Fig. 2).

Comparison of the T.R.C.H.I.I. and Latex Test on Clinical Material

A plot of the results from both techniques on 144 sera and 37 urines is shown in Fig. 3. There was a very satisfactory correlation between the two

methods ($r=0.8942$: $p < 0.001$), despite the use of anti-sera to whole fibrinogen in the T.R.C.H.I.I.

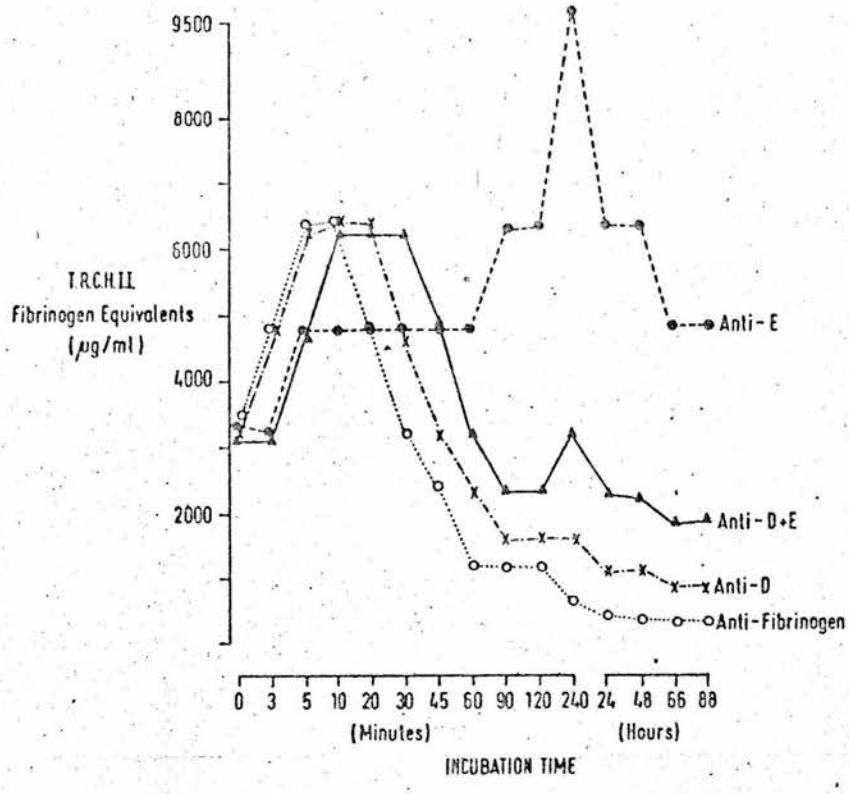

FIG. 2.

Results of the T.R.C.H.I.I. on in vitro human fibrinogen digests using antisera prepared to whole human fibrinogen, fragment D and fragment E.

Non-specific Latex particle agglutination

Less than 1% of 120 sera obtained from healthy blood donors agglutinated the latex particles coated with normal rabbit IgG. However, in patients with rheumatoid arthritis this rose to 25%: the highest titre recorded being 1/8. In urine specimens, whether from healthy subjects or patients with glomerulonephritis, this rose to 50% in fresh specimens but could be reduced to 25% after freezing, thawing and centrifugation. The highest titre recorded was 1/64. Complete removal of this non-specific latex agglutinating material was possible if 1 volume of urine was incubated with 1 volume of normal rabbit IgG coated latex for 30 minutes at $4^{\circ}C$.

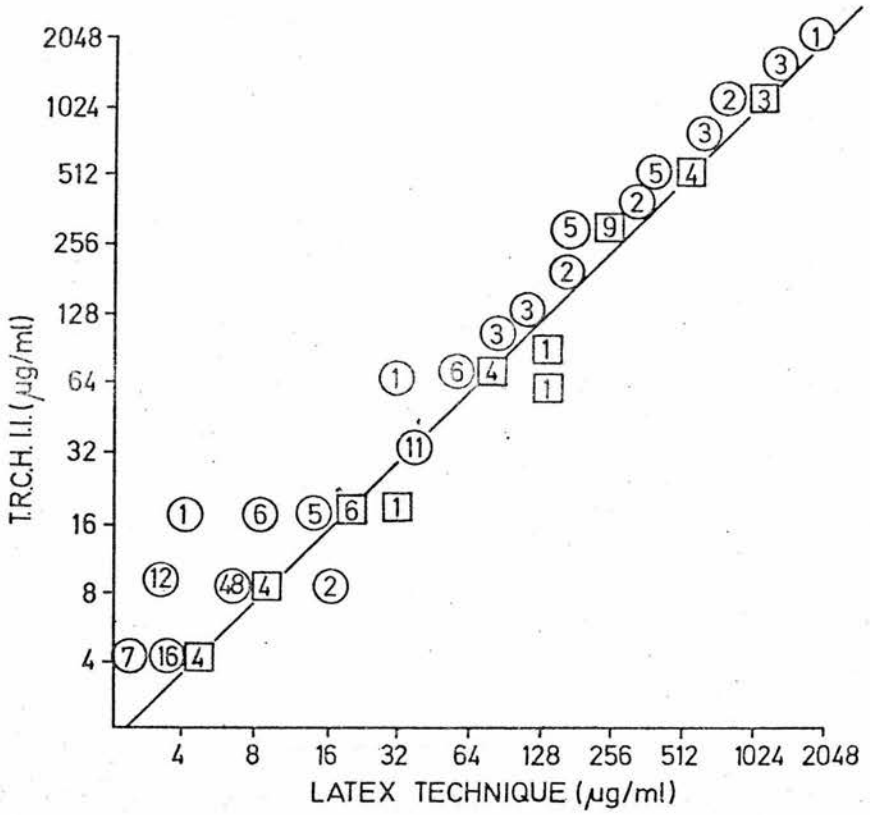

FIG. 3.

A correlation between the Latex test and T.R.C.H.I.I. on the F.D.P. values in sera (open circles) and urine (squares) (r=0.894 : p 0.001)

DISCUSSION

The results of this study suggest that the Thrombo-Wellcotest reagent appears to be a valuable approach towards a rapid 'one-off' bedside semiquantitation of serum F.D.P. and could, therefore, be readily introduced to samller non-specialised laboratories. The presence of non-specific latex agglutinating substances in sera is unlikely to be of material significance as in our experience it has not been recorded above a titre of 1/8 which corresponds to an F.D.P. level of only 32µg/ml fibrinogen equivalents.

At the present time we regard the Thrombo-Wellcotest as essentially unsuitable for the assay of urinary F.D.P. The high incidence of the non-specific latex agglutinating substance(s) would necessitate a pre-absorption

step prior to assay. Moreover, the serial dilution procedure is particularly laborious, compared to the microtitre technique used for the T.R.C.H.I.I. This latter feature is of great practical importance as it has been shown that if the quantitation of urine F.D.P. is proved to be relevant in the management of patients with certain forms of renal disease then large batches of assays are required on individual patients (12).

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. Pamela Pitcher, of Wellcome Reagents Limited for supplying the latex preparations and anti-sera to fragments D and E. This research programme was supported by a grant from the Scottish Hospital Endowments Research Trust.

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URINARY EXCRETION OF HETEROPHILE (SHEEP) HAEMAGGLUTININS AND FIBRIN/FIBRINOGEN DEGRADATION PRODUCTS FOLLOWING RENAL HOMOTRANSPLANTATION AND IN PROLIFERATIVE GLOMERULONEPHRITIS

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INTRODUCTION

A variety of serological responses to allotransplantation have been reported in man, including a rise in the titre of serum heterophile haemagglutinins, directed against sheep, guinea-pig and rat erythrocytes, after skin and renal transplantation.[1-3] The haemagglutinins were in the IgG and IgM fractions, did not appear to be the Forssman or Paul-Bunnel-type and the peak titres were thought to be associated with clinical and biochemical evidence of graft rejection. The mechanisms responsible for this phenomenon are not known, but it has been proposed that it might represent an immune response to altered tissue antigens arising from the rejection injury.[3] More recent studies have failed to confirm all aspects of these observations; in particular, the association between renal allograft rejection and peak titres of serum heterophile antibody has been regarded as coincidental.[4-6]

Within the last two years several investigations have shown that whereas the assay of another non-specific and presumptive marker of immune damage, fibrin/fibrinogen degradation products (F.D.P.), in the serum of patients with renal transplant and glomerulonephritis gave indifferent evidence of disease activity, the urinary F.D.P. content appeared to be a highly sensitive index of both activity and response to therapy.[7-9] Based on this principle, and the possibility that heterophile haemagglutinins might be directed at altered glomerular antigens, a study was established to detect and semiquantitate sheep haemagglutinins in urine rather than serum from patients with proliferative glomerulonephritis and those following renal homotransplantation, and

to examine their correlation with the urinary F.D.P. content.

PATIENTS AND METHODS

One hundred presumed healthy colleagues (50 male and 50 female; aged 18-58 years), were used as controls. Their urine specimens were obtained at random throughout a working day. Fifteen patients with proliferative glomerulonephritis and ten following renal homotransplantation were studied. Aliquots of urine from 24 hour collections were obtained from all in-patients, whereas out-patients sent early morning specimens through the postal services. All samples were dialysed against tap water, concentrated overnight with polyethylene glycol, centrifuged and stored at -36°C until assayed. Urinary F.D.P. content was determined by the tanned red cell haemagglutination inhibition immunoassay (T.R.C.H.I.I.), using glutaraldehyde fixed human red cells.[10] The urinary heterophile haemagglutinin titre was established, using glutaraldehyde fixed 'high reacting' sheep red cells.[10,11] Samples were serially diluted in distilled water. The system of scoring titres arithmetically, introduced by Race and Sanger,[12] was used to summarise and analyse the data. Both immunoassays were performed by independent operators who had no prior knowledge of the source of the test samples or of each other's results. The total protein content of the urinary concentrates was performed by the biuret method.[13]

RESULTS

Urinary sheep cell haemagglutinins were not detected in the 100 normal subjects studied, nor did the F.D.P. content exceed 0.25 ug/ml. In a selection of 60 urines, obtained from patients with proliferative glomerulonephritis and following renal transplantation, there was a significant positive correlation between the heterophile haemagglutinin titre and the F.D.P. content (Fig. 1). Serial studies performed on individual patients revealed a general tendency for periods of high heterophile haemagglutinin excretion to coincide with similar excesses of F.D.P. excretion. Some urine specimens, however, contained low F.D.P. and high heterophile haemagglutinin, and vice versa. Purified preparations of F.D.P. fragments D and E (kindly supplied by Doctor P. Gaffney) did not agglutinate sheep erythrocytes. No correlation was demonstrated between the heterophile haemagglutinin titre and total protein content in the urines studied ($r = 0.1796$: p 0.1).

Serial urine samples on 6 post-renal transplant patients studied continuously for 10-42 days demonstrated that the heterophile haemagglutinin titres and F.D.P. excretion provided parallel information on the detection of acute and sub-acute rejection, and the response to subsequent treatment. An example is shown in Fig. 2.

The responses to the administration of indomethacin in a group of 7 patients with active proliferative glomerulonephritis was also studied. In 5 patients there was a fall in F.D.P. and heterophile haemagglutinin excretion during indomethacin administration, although the latter was consistently more slow to respond. In all these patients there was biochemical evidence of improvement or stabilisation

of/

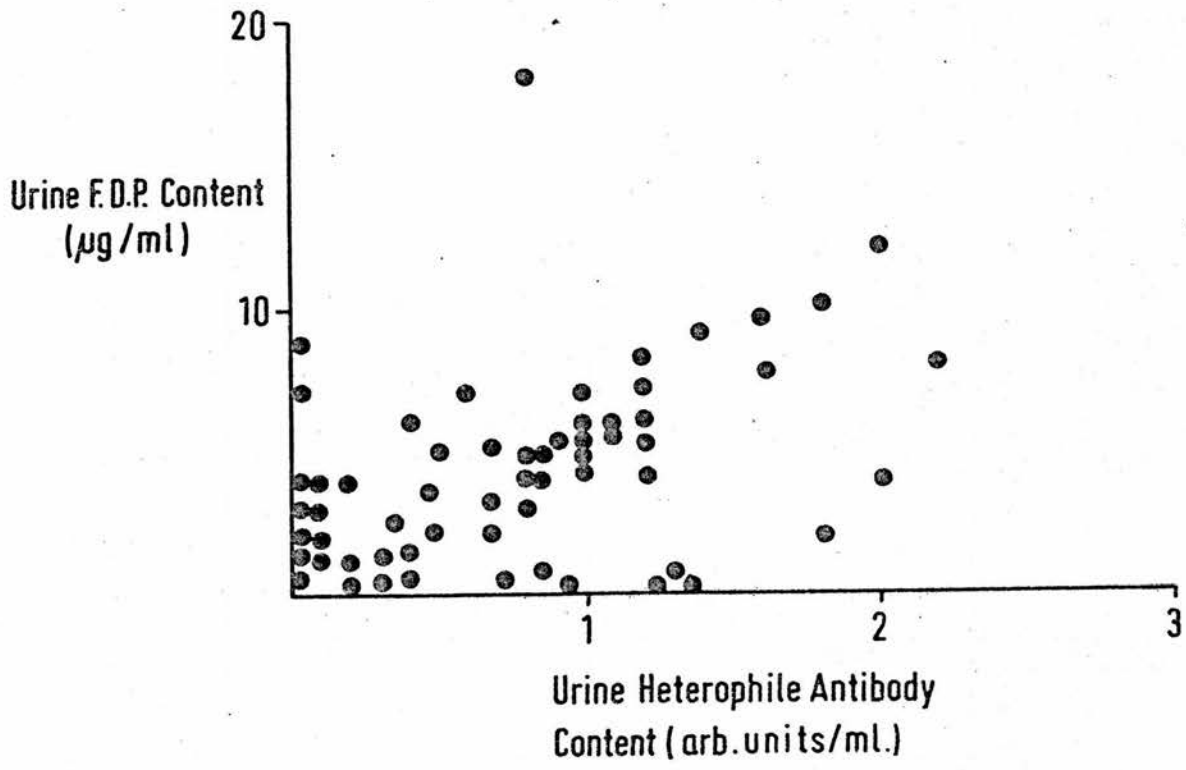

FIG. 1.

Correlation between F.D.P. and heterophile (sheep) antibody content in 60 selected urines obtained from patients with proliferative glomerulonephritis and following renal homotransplantation (r= 0.4891, p 0.001)

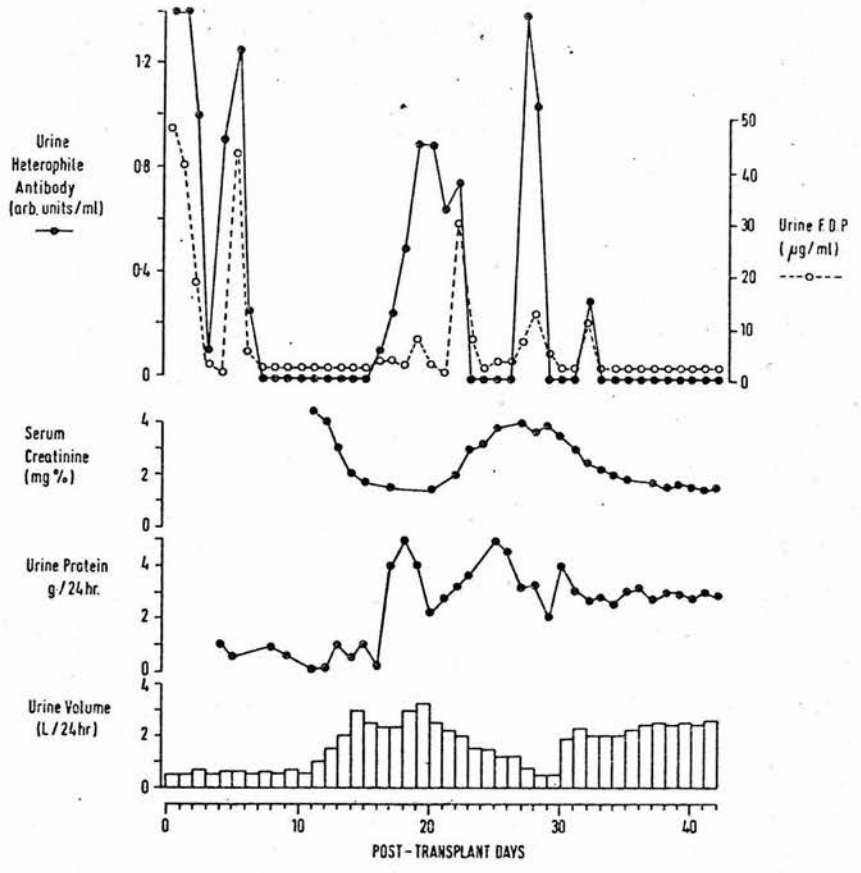

FIG. 2.

Urine volume, heterophile (sheep) antibody, F.D.P. and total protein content, and serum creatinine in a patient following renal homotransplantation. Clinical evidence of ~~a hyperacute~~ rejection was apparent by the 22nd. day.

of renal function. An example of this type of dual response is shown in Fig. 3. One of the 7 patients in this group responded quite differently: no evidence of a heterophile haemagglutinin response occurred despite a marked reduction in the F.D.P. excretion (Fig. 4). It was noted that this patient's renal function continued to deteriorate during indomethacin administration; and renal transplantation was undertaken several months later. Of further interest was the observation that a second patient in whom indomethacin failed to ellicit a fall in urine F.D.P. also showed no diminution of heterophile haemagglutinin excretion.

DISCUSSION

Previous studies have demonstrated a significant correlation between the activity of glomerular disease, as assessed biochemically and histologically and the excretion of urinary F.D.P. in proliferative glomerulonephritis,[9,14] and during the rejection of renal homografts.[7,15] The preliminary study described above suggests that in these two patient groups similar information may be obtained from serial assays of the urinary heterophile haemagglutinin content. This general conclusion is supported by a significant correlation between urinary F.D.P. and heterophile haemagglutinin concentrations in 60 separate urines, the parallel information obtained from serial studies and the changes in both parameters during therapy. Moreover, just as a proportion of patients with active proliferative glomerulonephritis fail to reduce their F.D.P. excretion during indomethacin/

indomethacin administration,[9] the same appears to hold for the heterophile haemagglutinin excretion. A potentially important exception to this parallelism was found in one patient who showed diminishing F.D.P. excretion in response to indomethacin but continuing high urinary heterophile haemagglutinin excretion. The renal function in this patient progressively deteriorated. If this observation is confirmed, then a new subgroup of patients has been demonstrated in which the presence of intraglomerular fibrin may not be a key aetiological factor in the progressive damage associated with proliferative glomerulonephritis.

The mechanisms responsible for urinary heterophile haemagglutinin excretion in active proliferative glomerulonephritis and renal homograft rejection are as yet unknown. The data, presented, however, showing no correlation with the total protein excretion, and rapid responses to therapy which also appeared independent of total protein excretion, would suggest that its appearance is part of an active process, rather than arising from the passive glomerular leakage of serum proteins. While these preliminary observations require more detailed confirmatory studies, and should also be extended to other areas in nephrology, they already appear to have interesting theoretical and practical consequences. For the clinician, the study of heterophile haemagglutinin titres alone, or combined with urinary F.D.P. excretion, may provide yet another approach to the understanding of the pathogenesis and management of patients with proliferative glomerulonephritis and renal allograft rejection. Moreover, from a laboratory point of view, the assay of urinary F.D.P. using a haemagglutination inhibition immunoassay is

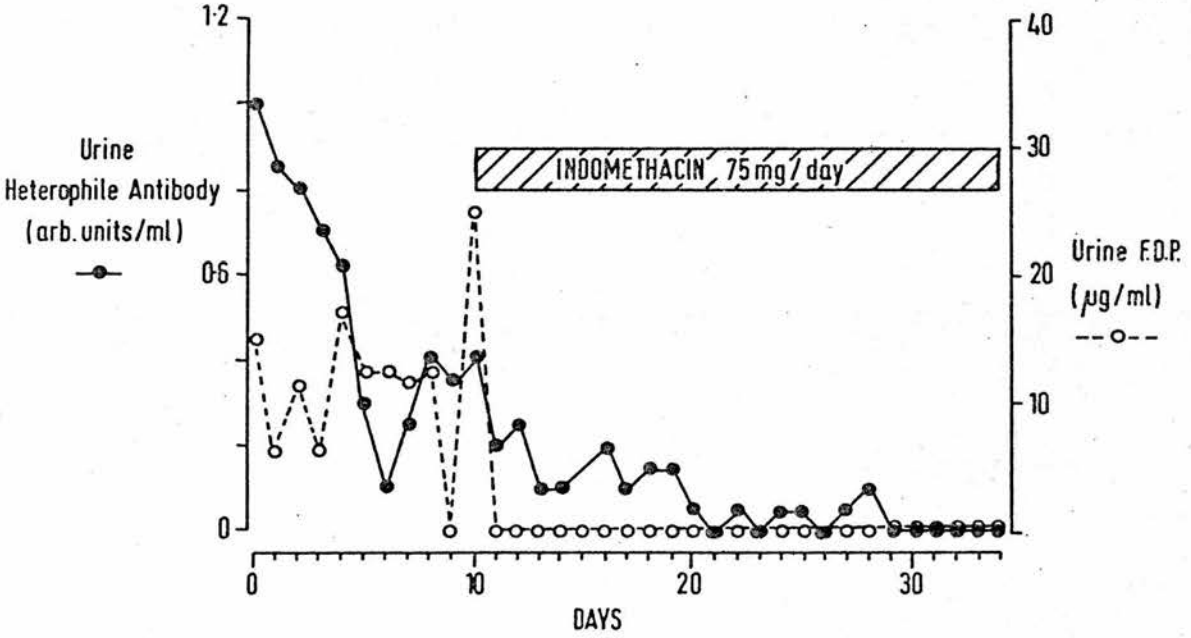

FIG. 3.

The pattern of urinary heterophile (sheep) antibody and F.D.P. excretion in a patient with proliferative glomerulonephritis whose renal function improved during indomethacin administration.

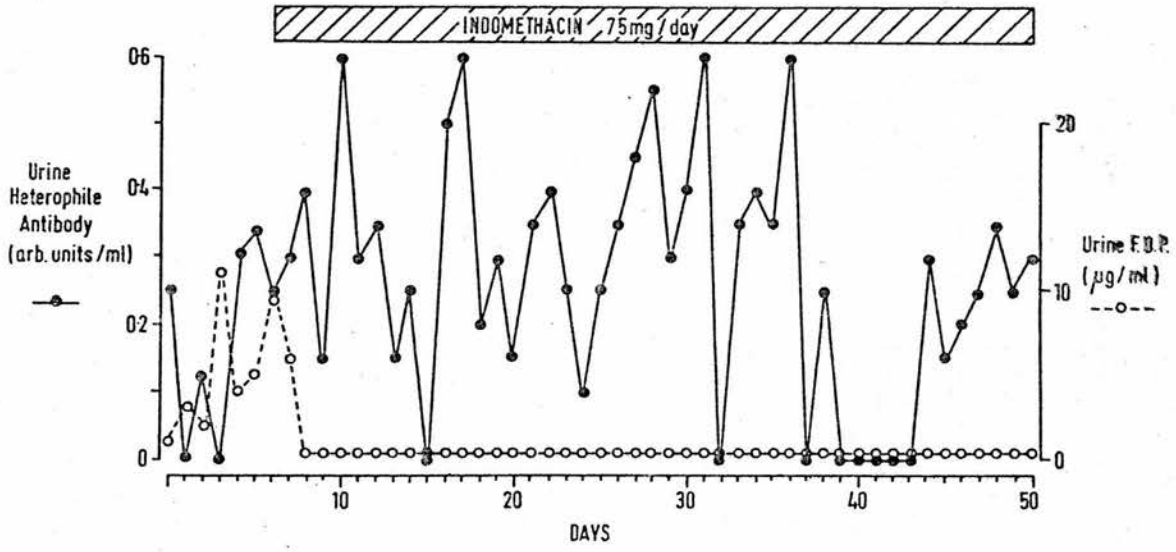

FIG. 4.

The pattern of urinary heterophile (sheep) antibody and F.D.P. excretion in a patient with proliferative glomerulonephiritis whose renal function continued to deteriorate during indomethacin administration.

laborious and time consuming whereas the heterophile haemagglutinin assay, being a direct agglutination reaction, is technically simpler, the reagents readily available and preliminary investigations in this laboratory suggest that automation should be straight forward.

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